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ABSTRACT

During the funding period (June 01 – May 05), we have completed the most Aims and subaims proposed in the proposal, i.e. to determine the anti-tumor and the pro-apoptotic activities of p202 in prostate cancer cells; to understand the molecular mechanisms underlying the p202-mediated anti-growth, anti-tumor, and potential pro-apoptosis activities in prostate cancer; and to test the anti-tumor activity of p202 in prostate cancer cells using preclinical gene therapy strategies and to determine the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer model. In an attempt to initiate a p202 clinical trial, we have communicated with the FDA and been informed that the human gene is preferable over murine one. Therefore, we have proceeded to clone a human counterpart of p202 gene, the AIM2 (absent in melanoma). We have obtained encouraging results on AIM2 that has the similar effect of p202 in anti-growth activity in prostate cancer cells in cell culture, and exhibits an anti-tumor growth activity in animals bearing mammary tumors. To further extend the specificity for both androgen receptor-positive and –refractory prostate cancers, we have newly developed a broad spectrum of robust prostate cancer-specific expression vector. We intend to test the therapeutic efficacy and specificity by using the broad spectrum of prostate cancer-specific promoter to drive the therapeutic gene, AIM2 in animals. In an attempt to discover more novel strategies to treat prostate cancer, emodin, which has shown a promising killing effect on prostate cancer cells.

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INTRODUCTION:

The interferon family (IFN) is composed of three classes: α, β an γ (1). The IFN family not only plays an integral role in host defense system against certain tumors and foreign antigens such as viruses, bacteria and parasites; but also possesses immunomodulatory and cell growth-inhibitory activities. However, the molecular mechanisms involved in IFN's anti-tumor activity are remained elusive. In a recent study, several IFN-inducible proteins are implicated in the process of tumor suppression (2). Moreover, based on DNA analysis, 19 out of 95 IFN-inducible genes are differentially downregulated during prostate tumor progression (3). The anti-cellular function of IFNs has been attributed to their abilities to induce G_1 phase arrest in cell cycle (4-6). P202, an IFN-inducible gene is a primarily nuclear 52kd phosphoprotein, has been shown to have a growth retardation function that was presumably accomplished by its ability to bind several cell-cycle regulatory proteins such as E2Fs, AP1, NFκB and pRb, resulting in the failure of S phase entry (7-9). Using p202 as a therapeutic agent, we have demonstrated that the multiple anti-tumor activities in human cancer xenograft models including breast and pancreatic cancers (10-11). Tumor-bearing mice treated with liposome/p202 complex had suppression of tumor growth, inhibition of angiogenesis and metastasis. In an earlier study on human prostate cancer cells, we observed that augmented expression of p202 inhibits cellular proliferation and suppresses transformation phenotype *in vitro* (12). Our ultimate goal is to develop a gene therapy strategy that would specifically deliver p202 to the prostate cancer cells so that the "normal cells" would not be affected by such treatment. To accomplish our goal, three specific aims are proposed (see below). The success of those aims will constitute a scientific basis for p202-associated anti-tumor effect on prostate cancer cells and will enable us to develop a novel p202 gene therapy strategy against prostate cancer.

Within the three-year funding period, we have succeeded to complete most of the Aims (and subaims). In an attempt to develop the p202 gene as a therapeutic gene for human clinical trials, we initiated a communication with the FDA and were told that the p202 gene is of mouse origin and it is not favorable for human clinical trials. Hence, to

circumvent the potential drawback of using murine p202 gene for clinical trials and to maintain the benefits of p202 therapeutic efficacy, we have screened human p202 family genes and identified the AIM2, a human IFN-inducible protein, as a potential therapeutic agent in place of p202. The structurally related human (AIM2, IFI16 and MNDA) and murine (p202, p203, p204 and D3) genes belong to the 200-family, a family consisting of IFN-inducible proteins (13-18). Since then, we have shown that AIM2 gene has exhibited many characteristics of p202, a manuscript is under preparation (portion of figures are included as Appendix 1 in this final report).

BODY:

A. SPECIFIC AIMS: (NO CHANGES)

Specific Aim 1: To determine the anti-tumor and the pro-apoptotic activities of p202 in prostate cancer cells.

a. determine the anti-tumor and the pro-apoptotic activities of p202 in prostate cancer cells;

b. determine pro-apoptotic activity of p202 in response to therapeutic agents, e.g. TNFα.

Specific Aim 2: To understand the molecular mechanisms underlying the p202-mediated anti-growth, anti-tumor, and potential pro-apoptosis activities in prostate cancer.

a. determine the effect of p202 on G1/S cell cycle regulators in prostate cancer cells;

b. determine the effect of p202 on G2/M cycle regulators in prostate cancer cells.

Specific Aim 3: To test the anti-tumor activity of p202 in prostate cancer cells using preclincal gene therapy strategies and to determine the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer model.

a. test the anti-tumor activity of p202 gene for tumors induced by s.c. injection;

b. test the anti-tumor activities of p202 gene for orthotopic prostate model;

c. develop a prostate-specific gene therapy strategy.

B. STUDIES AND RESULTS:

In this final report, we have completed the **Specific Aim 1 a, Specific Aim 2 a to b, and Specific Aim 3 a to c** in the funding period. As mentioned above, in an attempt to develop the p202 gene as a therapeutic gene for human clinical trials, we initiated a communication with FDA and were told that the p202 gene is of murine origin and therefore, it is not appropriate for human clinical trials. In the last report, we have shown that AIM2 (Absent In Melanoma), a human homologue of p202 gene, has an anti-growth activity. Here, we report that AIM2 exhibits an anti-tumor activity on human breast cancer in an orthotopic mouse model (Appendix 1, Fig.1). Using luciferase report assay, we have demonstrated that, AIM2 expression as expected to be similar to p202, can repress NF-κB-mediated transcription activation in response to TNFα and p65-activated transcription, respectively (Appendix 1, Fig. 2 and 3). Therefore, human AIM2 may suppress tumor growth through a similar mechanism of p202.

To further investigate the growth inhibitory effect of AIM2, we have established stable clones of AIM2 by an inducible tetracycline regulatory system (Tet-off) *in vitro* (Appendix 1, Fig. 4). In addition, we observed that AIM2 expression under inducible tetracycline regulatory system (Tet-off) decreases the tumor volume on human breast cancer in an orthotopic mouse model (Appendix 1, Fig. 5). Thus, AIM2, like mouse p202 possesses an anti-tumor activity; and could be a potential candidate for future human clinical trials.

For patients with advanced or metastatic prostate cancer that is initially androgen-dependent (ADPC), hormonal deprivation therapy (ADT) is the primary choice. However, this modality eventually fails because prostate cancer frequently develops to an androgen-independent state within 2 years (19,20). Androgen-independent prostate cancer (AIPC) is an untreatable form of prostate cancer in which the normal dependence on androgen for growth and survival has been bypassed (21). AIPC is a lethal form of prostate cancer that progresses and metastasizes. In the prostate cancer gene therapy setting, prostate specific promoters, such as prostate-specific antigen (PSA) (22-24) and probasin (PB) (25-28), have been recently developed and tested by many groups

including us. We have recently developed a robust prostate cancer promoter composite using the hTERTp promoter as a base in combination of TSTA and WPRE. Telomerase is an RNA-dependent DNA polymerase that synthesizes telomeric DNA. The telomerase holoenzyme is composed minimally of a constitutively expressed, template-containing RNA subunit and a catalytic protein subunit (human telomerase reverse transcriptase, hTERT) (29). Telomerase expression is highly elevated in malignant tumors and cancer cell lines uncluding prostate cancer cells but not in normal tissue (30). Recently, the hTERT promoter (hTERTp) has been used for cancer gene therapy (31-33). However, the activity of this unmodified hTERTp is much weaker than that of the commonly used CMV promoter (31,34,35). To augment the transcriptional activity of hTERTp, we have recently developed a robust prostate cancer-specific expressing vector using the two-step transcriptional amplification (TSTA) system (36,37). This robust expression systems allows the therapeutic gene to be selectively expressed in prostate cancer cells in a level that is comparable to that of CMV promoter, yet its expression level in normal tissues and cell lines are virtually non-detectable compared to that of CMV promoter (please see Appendix 3 for detailed description). This TSTA-derived expression vector will be useful for prostate cancer targeting gene therapy.

In addition to development for prostate cancer-specific gene therapy, in the funding period, we also identified a small molecular, emodin (1,2,8-trihydroxy-6-methylanthraquinone), a natural compound extracted from Rheum palmatum, can directly target AR to suppress prostate cancer cell growth *in vitro*. Our results showed that emodin inhibits AR transcriptional activity by preventing AR nuclear translocation. Emodin treatment results in decreased association of AR and heat shock protein 90 (hsp90) but increased association of AR and MDM2, thus inducing AR degradation in a ligandindependent manner. Most importantly, we showed that, through targeting AR, emodin could suppress prostate cancer cell growth in vitro and prolong the survival of prostate cancer–producing C3(1)/SV40 transgenic mice *in vivo*. Our work indicates a new mechanism for the emodin-mediated anticancer effect and justifies further investigation of emodin as a therapeutic and preventive agent for prostate cancer (please

see Appendix 2 in detail).

KEY RESEARCH ACCOMPLISHMENTS:

SPECIFIC AIMS

- We have completed Specific Aim 1 a, Specific Aim 2 a to b, and Specific Aim 3 a to c.
- AIM2 possesses anti-cancer/anti-tumor activities in vitro and in vivo.
- We have developed a robust prostate cancer-specific promoter for both androgen dependent and independent prostate cancers.

REPORTABLE OUTCOMES AND PERSONNEL

A. REPORTABLE OUTCOMES:

PUBLICATION

1. Wen, Y., Giri, D., Yan, D.-H., Spohn, B., Zinner, R. G., Xia, W., Thompson, T.C., Matusik, R.J. and Hung, M.-C Prostate specific anti-tumor activity by probasin promoter-directed p202 expression. *Mol. Carcino.* 37:130-137, 2003.
2. Cha, T.-L., Qiu, L., Wen, Y. and Hung, M.-C. Emodin downregulates androgen receptor and inhibits prostate cancer cell growth. *Cancer Res.* 65:2287-2295, 2005.

GRANT APPLICATION

Under the support of DAMD17-01-0-0071, we have submitted a grant entitled "Targeted gene therapy for androgen dependent and independent prostate cancer" (log number: PC051055) for 02/08/05 deadline. (Appendix 3 due to the large size, only the scientific body part of grant application is included for the Final Report)

B. PERSONNEL

Principal Investigator: Mien-Chie Hung, Ph.D.

Co-Project Investigator: Lin Qiu, Ph.D.

Co-Project Investigator: Dipak Giri, Ph.D.

Co-Project Investigator: Yong Liao, Ph.D.

Ph.D. Graduate Student: Yan Li, M.D.

Ph.D. Graduate Student: Xiuping Liu

Ph.D. Graduate Student: Zhenming Yu

CONCLUSIONS AND SIGNIFICANCE:

We have completed the Specific Aim 1 a, Specific Aim 2 a to b, and Specific Aim 3 a to c in the funding period. In an attempt to develop the p202 gene as a therapeutic gene for human clinical trials, we initiated a communication with the FDA and were told that the p202 gene is of mouse origin and it is not favorable for human clinical trials. Hence, to circumvent the potential drawback of using murine p202 gene for clinical trials and to maintain the benefits of p202 therapeutic efficacy, we use the AIM2, a human IFN-inducible protein, as a potential therapeutic agent in place of p202. human AIM2 may suppress tumor growth through a similar mechanism of p202. Thus, AIM2 could be a potential candidate for future human clinical trials. To further specially target prostate cancer, we have developed a prostate cancer-specific expression vector for both androgen dependent and independent prostate cancers using TSTA system to augment the expression of gene of interested. This expression vector is highly specific for prostate cancer and will be conveniently applied for development of prostate cancer-specific gene therapy.

We greatly appreciate the funding opportunity provided by Department of Defense on prostate cancer. We have achieved most of our original goals. Due to the practical reason that p202 is of mouse origin, we have further developed other directions to fulfill our goals to develop targeted gene therapy for prostate cancer, namely identification of human AIM2 and its anti-cancer activity; and development of a robust prostate cancer-specific expression vector using the two step TSTA system. In addition, we have also developed a small molecular, emodin, a natural compound extracted from Rheum palmatum that my serve as an anti-prostate cancer agent (Appendix 2).

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APPENDICES

APPENDIX 1: Figures and Legends

APPENDIX 2: Reprint--Cha, T.-L., Qiu, L., Wen, Y. and Hung, M.-C. Emodin downregulates androgen receptor and inhibits prostate cancer cell growth. *Cancer Res.* 65:2287-2295, 2005.

APPENDIX 3: "Targeted gene therapy for androgen dependent and independent prostate cancer" (Log number: PC051055) Scientific Body portion only

Appendix 1: 5 Figures

Fig.1 AIM2 exhibits an anti-tumor effect on human breast cancer cell in an orthotopic mouse model. Six week-old female nude mice (ten mice/group) were inoculated with $2x10^6$ MDA-MB-435 breast cancer cells under mammary fat pad. Intra-tumoral gene therapy with AIM2/Liposome (20μg/8μl, every other day) started after tumors being established. Tumor sizes were measured with a caliper twice a week. The tumor volume was calculated using the formula: Volume SxSx*L*/2 where *S* is the short length of the tumor in cm and *L* is the long length of the tumor in mm ($p<0.01$).

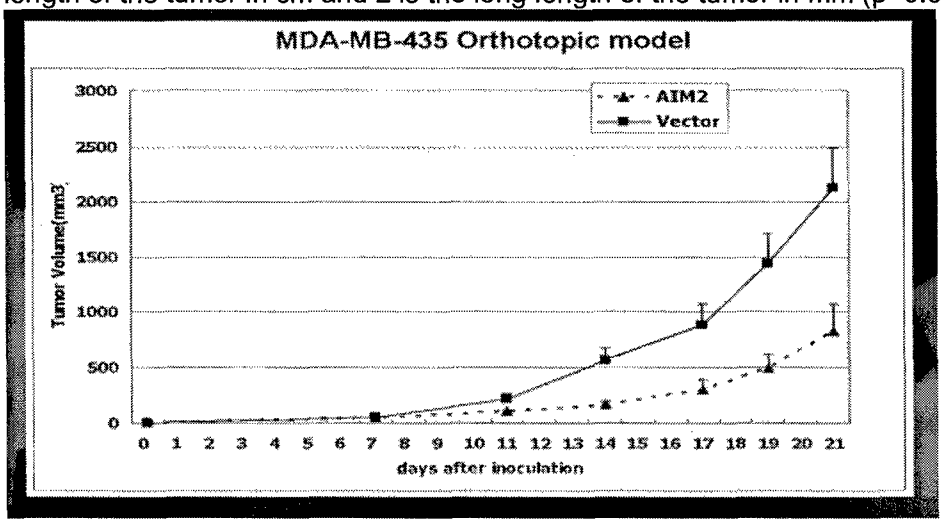


Fig. 2 AIM2 expression represses NF-κB-mediated transcription activation in response to TNF-α. κB-Luciferase reporter gene (0.2 μg) and CMV-Flag-AIM2 (0, 0.5, or 1.5 μg) were cotransfected into MDA-MB-453 and MDA-MB-435 cells. Thirty-six hours after transfection, cells were either left untreated or stimulated with TNFα (20ng/ml) for 6 hours. The relative luciferase activity in IκB-Luciferase expression was calculated by setting κB-Luciferase expression in the absence of TNFα and AIM2 as 100%. It is worthwhile to mention that the NF-κB activity in the presence of AIM2 is even lower than the basal activity without TNFα stimulation. It suggests that AIM2 strongly inhibits NF-κB activity including both basal level and TNFα-induced NF-κB activity.

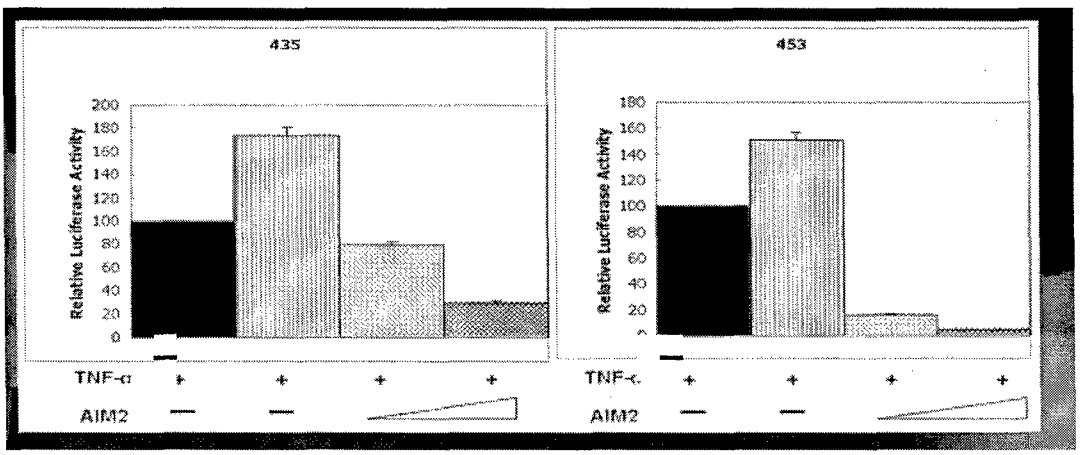


Fig. 3. AIM2 expression represses p65-activated transcription. MDA-MB-453 and MDA-MB-435 cells were cotransfected with κB-luc and NF-κB (p65) expression vectors. The inhibitory activity of AIM2 on the induction of IkB promoter activity by p65 was assessed by cotransfection with AIM2 expression vector. Luciferase activity was measured 48 hrs after transfection. The data represent an average of two independent experiments after normalization.

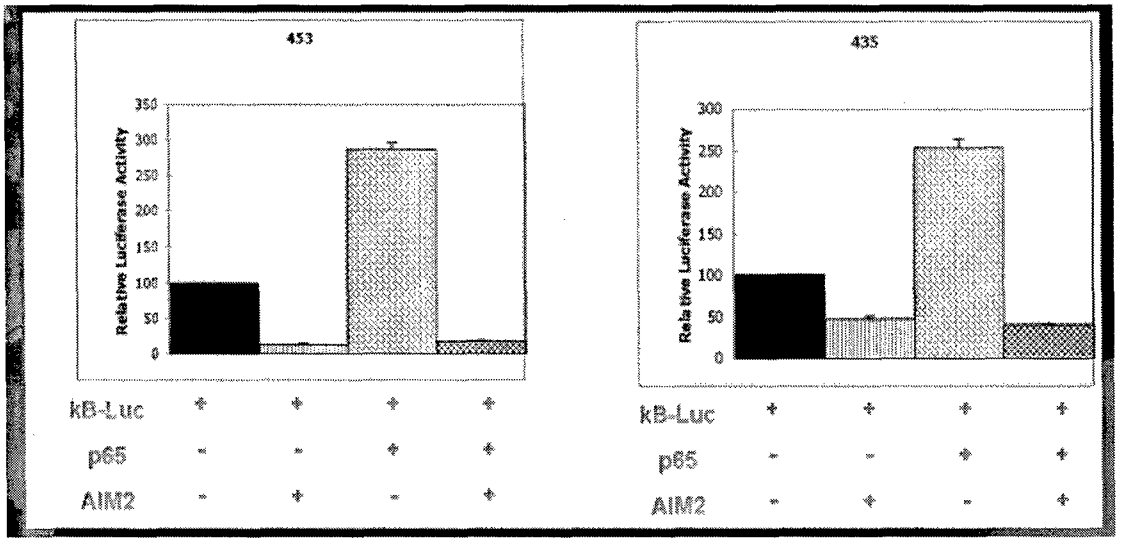


Fig. 4. The AIM2 suppressed breast cancer cells growth under inducible condition. Two stable clones, #13 and #25, of MCF-7 under tetracycline regulation system (tet-off) were selected. AIM2 expression levels were detected in panel (A). Cells were kept in medium containing with doxycycline (1mg/ml). After seven days remove of doxycycline in medium, cells were lysed and Western block with anti-Flag antibody to detect AIM2 expression level. (B) The growth rate was measured by cell number versus time of growth. $2x10^4$ cells were plated under either with or without doxycycline, cells were collected and accounted every other days. Cell numbers were plot in panel B.

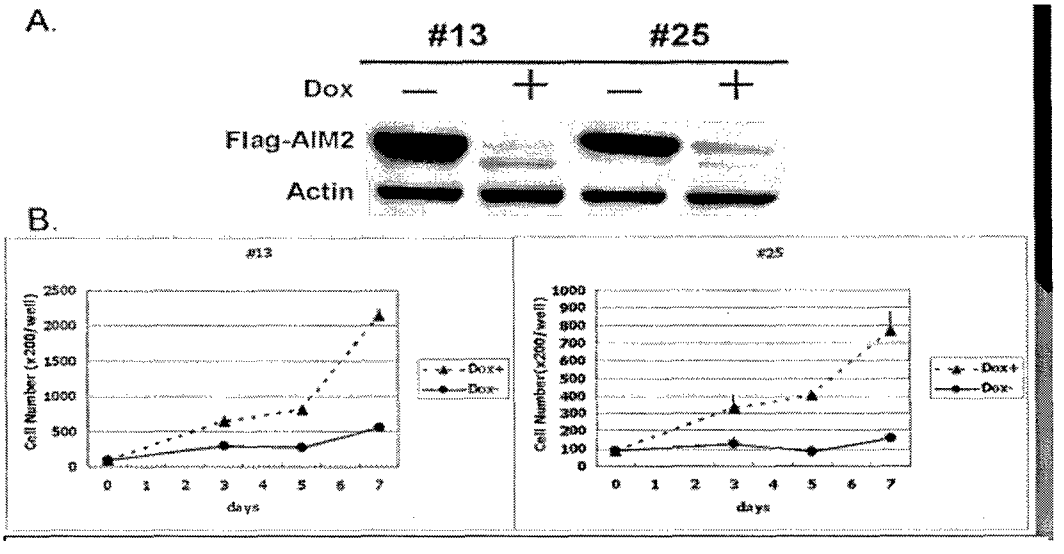
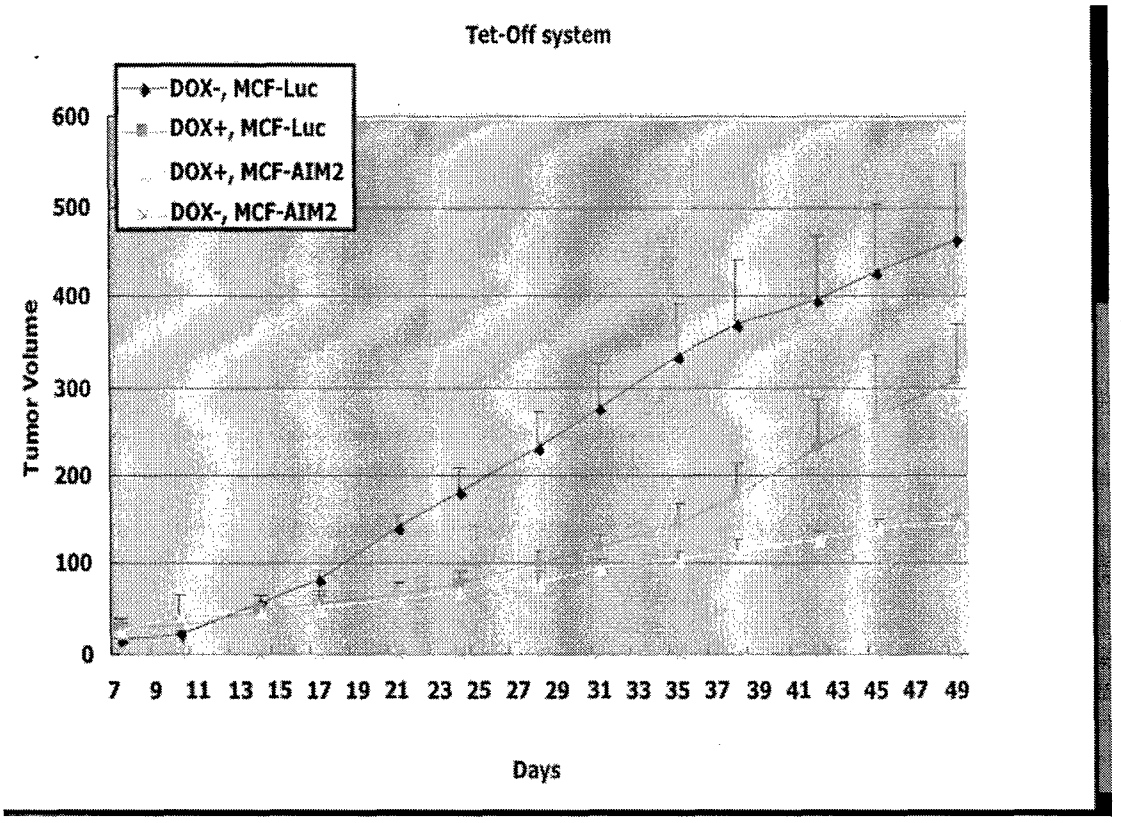


Fig. 5. Tet-off AIM2 exhibits an anti-tumor effect on human breast cancer cell in an orthotopic mouse model. Six week-old female nude mice (ten mice/group) were inoculated with $2x10^6$ cells of MCF-7 stable clones which gene expression under tetracycline regulation system (MCF-Luc: stably express luciferase after removal of textracycline/Doxicycline from media; MCF-AIM2: stable clone of AIM2 tet-off system). The mice were fed with sucrose water with/without Doxicycline (2mg/ml). Tumor sizes were measured with a caliper twice a week. The tumor volume was calculated using the formula: Volume SxSx*L*/2 where *S* is the short length of the tumor in cm and *L* is the long length of the tumor in mm (*bar, SE*).



Emodin Down-regulates Androgen Receptor and Inhibits Prostate Cancer Cell Growth

Tai-Lung Cha,[1,2,3] Lin Qiu,[1] Chun-Te Chen,[1] Yong Wen,[1] and Mien-Chie Hung[1,2]

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Abstract

Hormone-refractory relapse is an inevitable and lethal event for advanced prostate cancer patients after hormone deprivation. A growing body of evidence indicates that hormone deprivation may promote this aggressive prostate cancer phenotype. Notably, androgen receptor (AR) not only mediates the effect of androgen on the tumor initiation but also plays the major role in the relapse transition. This provides a strong rationale for searching new effective agents targeting the down-regulation of AR to treat or prevent advanced prostate cancer progression. Here, we show that emodin, a natural compound, can directly target AR to suppress prostate cancer cell growth *in vitro* and prolong the survival of C3(1)/SV40 transgenic mice *in vivo*. Emodin treatment resulted in repressing androgen-dependent transactivation of AR by inhibiting AR nuclear translocation. Emodin decreased the association of AR and heat shock protein 90 and increased the association of AR and MDM2, which in turn induced AR degradation through proteasome-mediated pathway in a ligand-independent manner. Our work indicates a new mechanism for the emodin-mediated anticancer effect and justifies further investigation of emodin as a therapeutic and preventive agent for prostate cancer. (Cancer Res 2005; 65(6): 2287-95)

Introduction

Prostate cancer is the most common malignant disease and the second leading cause of death in U.S. male cancer patients. Despite that diagnosis is earlier than in the past, the incidence and mortality rates of this cancer are still increasing steadily. About 220,900 cases diagnosed and 28,900 deaths were attributed to the disease in 2003 (1), and inevitably, 29,900 men are expected to have die of this disease in 2004 (2). On this devastating disease with tremendous impact on public health, unfortunately, the effective treatment options are limited and metastatic disease frequently develops even after potentially curative surgery or radiation therapy (3–5). Besides focusing on early diagnosis and treatment of this long-term and multistep malignant disease, prevention may be an alternative and more effective approach.

A recent and exciting prevention trial for prostate cancer has been done to show that finasteride, a 5-α reducatase inhibitor that inhibits the conversion of testosterone to a more potent androgen, dihydrotestosterone, has the chemopreventive effect for prostate cancer development (6). Their results support that prevention could be a right direction and strategy while dealing with prostate cancer, but this study points out that finasteride also increases the risk of high-grade prostate cancer. One possible explanation for the outcome may have resulted from the fact that finasteride reduced the intraprostatic dihydrotestosterone level that created an environment more beneficial for those less androgen-dependent, high-grade cancers to grow (7, 8). Consistent to this notion, previous reports showed that men who developed prostate cancers with low testosterone levels have higher Gleason grades and worse outcomes than those with normal testosterone levels (9–11). Thus, although the clinical trial was successful, the results do not provide a clear resolution for patients and physicians to choose finasteride as a preventive agent due to the potential high risk for development of high-grade prostate cancer, which is associated with much higher mortality rate.

Prostate cancer depends on androgen receptor (AR) to mediate the effect of androgen on tumor initiation and progression (12). The standard hormone therapy for prostate cancer aims at inactivating AR transcriptional activity by androgen deprivation (through surgical or medical castration) or androgen blockade (with AR antagonists; refs. 13, 14). The same concept has been applied to prostate cancer prevention. However, for those advanced prostate cancer, this response is temporary; as disease progresses, almost all prostate cancers eventually become androgen independent. More and more evidence suggest that hormone therapies for prostate cancer may promote the phenotypic progression of those tumor cells that are able to survive the acute period of the therapy (15, 16). Although prostate cancer uses various schemes to subvert normal restraints on cell growth along with deprivation of androgen, a common feature among the diverse schemes is that the AR is still expressed and required for androgen-independent prostate cancer cell growth (17–20). These tumors are androgen independent but seem to remain AR dependent. Thus, it is important to develop new compounds that can inhibit AR function in an alternative, ligand-independent manner.

Many phytochemicals derived from plants, such as genistein and curcumin, have been shown to possess substantial anticancer activities in prostate cancer, and clinical trials using these phytochemicals to prevent prostate cancer are ongoing (21). In the present study, we found that emodin (1,2,8-trihydroxy-6-methylanthraquinone), a natural compound extracted from *Rheum palmatum*, has more potent anticancer activity of prostate cancer than genistein and curcumin. Our results showed that emodin inhibits AR transcriptional activity by preventing AR nuclear translocation. Emodin treatment results in decreased association of AR and heat shock protein 90 (hsp90) but increased association of AR and MDM2, thus inducing AR degradation in a ligand-independent manner. Most importantly, we showed that, through targeting AR, emodin could suppress prostate cancer cell growth

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in vitro and prolong the survival of prostate cancer–producing C3(1)/SV40 transgenic mice *in vivo*. These results indicate a new mechanism for the emodin-mediated anticancer effect and justify further investigation of emodin, a natural compound as a therapeutic and preventive agent for prostate cancer.

Materials and Methods

Cell Culture. LNCaP, PC3, DU-145, 293, and COS-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in serum-containing medium as recommended by the supplier. PC3-AR cells are a clonal cell line derived by stable transfection of PC3 cells with a plasmid containing the coding region of the human AR. PC3-neo cells were stably transfected with the same vector lacking the AR cDNA sequence. Medium for PC3-AR cells also contained the selective antibiotic G418 (400 μg/mL, Life Technologies, Inc., Gaithersburg, MD). For experiments requiring an androgen-depleted condition, cells were incubated in phenol red–free RPMI 1640 supplemented with 5% charcoal-stripped fetal bovine serum (c-FBS) for 1 day before initiation of the experiment.

Reagents and Plasmids. The synthetic androgen R1881 (Perkin-Elmer, Boston, MA) was dissolved in 100% ethanol and stored at −20°C for up to 1 month. Emodin (Sigma Co., St. Louis, MO), MG-132, AG1478, LY294002, U0126, curcumin, and genistein (Calbiochem, San Diego, CA) were dissolved in DMSO. Anti-AR antibody (15061, 15071), anti-MDM2 antibody, and anti-hsp90 antibody were purchased from PharMingen (San Diego, CA), Oncogene (San Diego, CA), and Santa Cruz Biotechnology (Santa Cruz, CA) respectively. Expression plasmids used were pSG5-AR, pcDNA3-MDM2, and pcDNA3.1-green fluorescent protein (GFP)-AR. pSG5-AR was generated by inserting the human AR cDNA into the *Eco*RI and *Bam*HI sites near the start and termination codons of expression vector pSG5. pcDNA3-MDM2 was generated as described previously (22). pcDNA3.1-GFP-AR was kindly provided by Dr. Zhengxin Wang (University of Texas M.D. Anderson Cancer Center, Houston, TX).

Proliferation and [^{3}H]Thymidine Incorporation Assays. Cells were seeded in 96-well microtiter plate overnight. Thereafter, cells were treated with different concentrations of emodin (0, 10, 20, or 40 μmol/L) and equal volume of DMSO as the control and then incubated for additional 24, 48, or 72 hours. The proliferation rates of the cell lines were analyzed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and [^{3}H]thymidine incorporation as described previously (23).

Reverse Transcription-PCR. Total RNA was extracted from cells and reverse transcription-PCR was done as per the manufacturer's instructions (SuperScript preamplification system, Life Technologies). The primers derived from the AR coding sequence (5′-CTCACCAAGCTCCTGGACTC-3′) and prostate-specific antigen (PSA) coding sequence (5′-GCAGCATT-GAACCAGAGGAG-3′) were used to amplify the AR and PSA transcripts, respectively. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal control were 5′-AGGTGAAGGTCGGAGTCAAC-3′ and 5′-TCCATTGATGACAAGCTTCCC-3′. Amplification was done on a Perkin-Elmer DNA cycler 480 for 35 cycles with denaturing at 94°C for 30 seconds, annealing at 58°C for 1.5 minutes, and extension at 72°C for 1.5 minutes.

Transient Transfection and Luciferase Assay. LNCaP and PC3 cells were plated 1 day before transfection at a density of 2×10^5 cells per well in six-well plates. LNCaP cells were cotransfected with a luciferase reporter plasmid (0.3 μg PSA-luc or probasin-luc), a β-galactosidase expression plasmid (0.2 μg CMV-β-gal), and expression plasmids with an empty vector (0.9 μg each) as indicated using liposomes. For PC3 cells, the procedure was the same, except that these cells were cotransfected with additional AR plasmid (0.3 μg pSG5-AR). After transfection, the cells were cultured in phenol red–free medium supplemented with 5% c-FBS in the absence or presence of the synthetic androgen R1881 (1 nmol/L) and various doses of emodin. Cell lysates were collected 48 hours after transfection, and the luciferase activity of each sample was measured using a luciferase assay kit (Promega, Madison, WI). β-galactosidase activity was determined to normalize variations in transfection efficiency.

Western Blot Analysis and Immunoprecipitation. LNCaP cells treated with emodin in presence or absence of 0.1 nmol/L R1881 were harvested, protein was extracted, and Western blots and immunoprecipitation were run as described previously (23). Primary antibodies against AR (1:500), MDM2 (1:1,000) were used to probe the blots. The intensity of the protein signal was quantitated by Bio-Rad PDQuest Image software (Hercules, CA).

Fluorescence Imaging and Fractionation. For immunodetection, treated cells were measured as described previously (22). Fluorescence imaging of living cells was done through an Axiovert 200 inverted fluorescence microscope (Zeiss, Germany). COS-1 cells were transiently transfected with chimeric GFP plasmid (pcDNA3.1-GFP-AR) and allowed to express chimeric protein for 24 hours. The cells were first analyzed without undergoing any treatment and then examined after the addition of different inhibitors for 30 minutes followed by the addition of 0.1 nmol/L R1881 into the same chamber. The same living cell was studied and recorded at different times. For cell fractionation, procedures were described previously.

Xenograft and Transgenic Mice Models. All animal experiments were done in accordance with institutional guidelines for animal welfare. PC3 (4×10^6) and PC3-AR (4×10^6) were injected s.c. into 5- to 6-week-old male athymic nude mice. One week after cell implantation, animals were randomized into two groups ($n = 10$ each). Each group was treated with i.p. bolus injections of either the drug vehicle (DMSO) or emodin (40 mg/kg) everyday. Tumors were measured with a caliper once a week, and their volumes were calculated using the formula: $\pi / 6 \times a \times b^2$, where a and b are the long and short diameters, respectively. Three pairs of C57BL/6J-TgN C3(1)/SV40 Tag-transgenic mice were purchased from Jackson Laboratory (Bar Harbor, ME) and the transgenic progeny was identified by PCR analysis of tail DNA isolated from 3-week-old litters using standard techniques. We treated 4-week-old mice (10 mice per group) with DMSO or emodin (40 mg/kg) everyday by i.p. injection. Each treated mouse received supplemental dosages at 140-day intervals, and the efficacy of the treatment was measured by body weight and survival. Additional groups of mice (six to seven mice per group) were given treatment as described above but sacrificed at age 21 weeks for histologic and Western blot analyses.

Immunohistochemical Staining. Immunohistochemical analysis for AR protein expression in tumor samples was done as described (24). Polyclonal antibody against AR was purchased from Upstate (Waltham, MA).

Statistics. All results from the *in vitro* experiments are presented as mean ± SE. Comparisons were made with Student's t test. SSPS software was used in all analyses.

Results

Emodin Inhibits Cell Proliferation of Androgen Receptor–Positive Prostate Cancer Cells. As an initial attempt to compare the effect of emodin on prostate cancer cells with other two well-characterized natural compounds, genistein and curcumin, we treated AR-positive LNCaP cells with low (10 μmol/L) and high (40 μmol/L) concentrations of emodin, genistein, and curcumin. Interestingly, the antiproliferative effect of emodin was more significant than genistein and curcumin even in low-dose range (Fig. 1*A* and *B*). Because AR-positive LNCaP cells respond and proliferate on androgen stimulation (25), we treated LNCaP cells with various concentrations of emodin in the presence of the synthetic androgen R1881. Emodin efficiently inhibited LNCaP cell proliferation and DNA synthesis stimulated by R1881 in a dose-dependent manner; meanwhile, the inhibition was also significant at low concentration (Fig. 1*C* and *D*). To further investigate the antiproliferative effect of emodin, we treated another prostate cancer cell line, DU-145, with various concentrations of emodin. DU-145 cells, which are derived from a brain metastasis, do not express the AR and grow independent of androgen. The AR-negative DU-145 cells are more resistant to the emodin-mediated antiproliferative effect than the AR-positive LNCaP cells (Fig. 1*E*). Although AR is not the only difference between LNCaP and DU-145

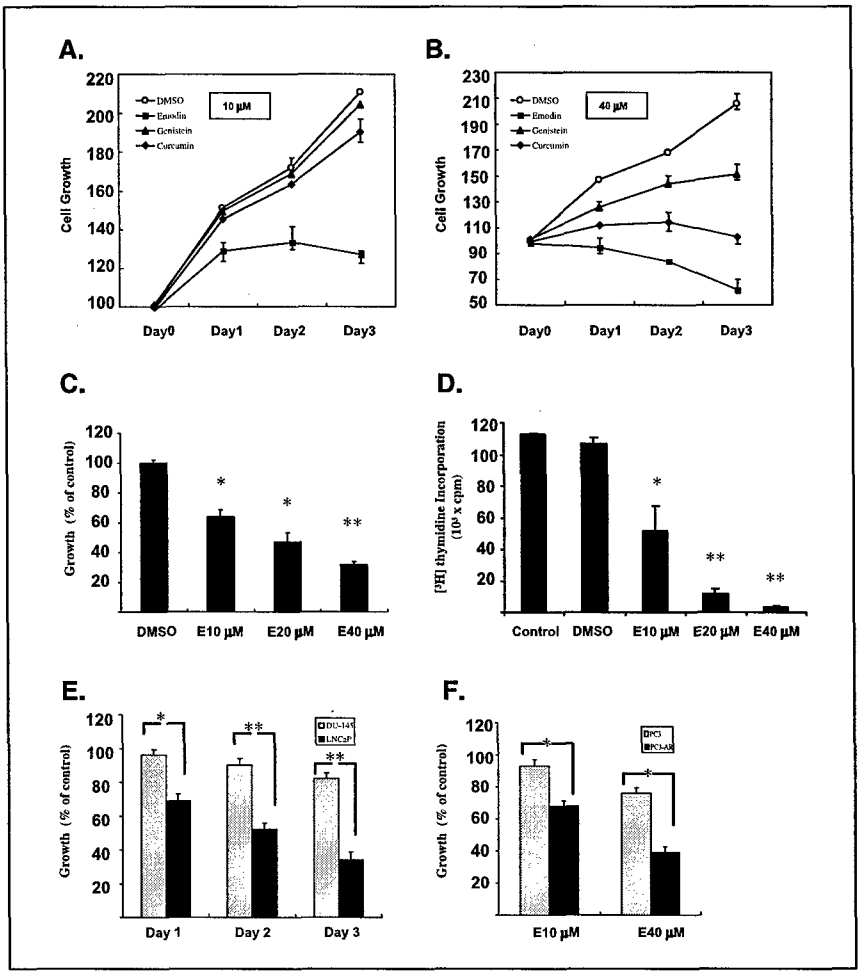

Figure 1. Effect of emodin on prostate cancer cells proliferation. *A* and *B*, LNCaP cells were treated with 10 or 40 μmol/L emodin, genistein, and curcumin for 24, 48, and 72 hours, respectively. Percentages of viable cells after treatment compared with control (DMSO treated defined as 100%) were determined by MTT assay. *C*, LNCaP cells were treated with DMSO and various concentrations [10 (*E10*), 20 (*E20*), and 40 (*E40*) μmol/L] of emodin in the presence of 1 nmol/L R1881 for 72 hours. Percentages of viable cells after treatment compared with control (DMSO treated defined as 100%) were determined by MTT assay. *, $P < 0.01$; **, $P < 0.001$, *t* test. *D*, LNCaP cells were treated with various concentrations of emodin in the presence of 1 nmol/L R1881. [^{3}H]Thymidine incorporation rates were done to measure DNA synthesis. *, $P < 0.01$; **, $P < 0.001$, *t* test. *E*, LNCaP and DU-145 cells were treated with DMSO or 40 μmol/L emodin for 24, 48, and 72 hours. Percentages of viable cells after treatment comparing to control were determined by MTT assay. *, $P < 0.01$; **, $P < 0.001$, *t* test. *F*, PC3 and PC3-AR cells were treated with DMSO and 10 or 40 μmol/L emodin for 72 hours. Percentages of viable cells after treatment were determined by MTT assay and compared with control. *, $P < 0.01$, *t* test.

prostate cancer cells, this result raises an interesting possibility that AR-positive prostate cancer cells may be more sensitive to emodin treatment. To extend this observation, we tested the effect of emodin on a pair of AR-negative and AR-positive prostate cancer cell lines, PC3 and PC3-AR. PC3 is a well-defined AR-negative prostate cancer cell line and PC3-AR is a clonal PC3 cell line stably transfected with AR. Thus, they have identical genetic background, except for the AR status. Again, the AR-positive PC3-AR cells are much more sensitive to emodin treatment than the AR-negative PC3 cells (Fig. 1*F*). Treatment with low-dose (10 μmol/L) emodin yielded a significant inhibition of PC3-AR cell growth by 35% within 72 hours compared with parental PC3 cell growth by 7% ($P < 0.01$); high-dose (40 μmol/L) emodin showed some inhibition of PC3 cell growth by 22% but more significant inhibition of PC3-AR cell growth by 60% ($P < 0.001$). The antiproliferative effect of the PC3-AR cells was evident already 24 hours after emodin treatment but not of PC3 cells (data not shown). Thus, this result is consistent with the results showed in Fig. 1*E* and supports the notion that AR-positive prostate cancer cells are more sensitive to emodin treatment.

Emodin Inhibits Androgen Receptor Transcriptional Activity and Nuclear Translocation. Because AR mediates the effect of androgen on cell proliferation and survival in LNCaP cells, and AR-positive prostate cancer cells are more sensitive to emodin treatment, we hypothesize that emodin inhibits the function of AR. To test this hypothesis, we investigated the effect of emodin on AR downstream target gene expression. Reverse transcription-PCR and Western blot analysis showed that expression of PSA, an AR-targeting gene, was down-regulated by emodin (Fig. 2*A* and *B*). To further test the effect of emodin on AR transcriptional activity, we transiently transfected LNCaP cells with PSA-luc and probasin-luc reporters, two well-characterized AR-targeting promoters. Both androgen-mediated PSA and probasin promoter activities were repressed in emodin-treated cells in a dose-dependent manner (Fig. 2*C*). The emodin-mediated repression of PSA and probasin reporter activities were also observed in PC3 cells with the cotransfection of AR-expressing vector, pSG5-AR plasmid and reporters (Fig. 2*D*). Without cotransfection of pSG5-AR, these two reporters will not respond to R1881 stimulation and emodin has no effect on their promoter activities in the AR-negative PC3 cells (data not shown). Thus, emodin inhibits the transcriptional activity of AR. The function of AR is closely related to its subcellular localization. Because emodin can inhibit AR transcriptional activity, we examined the effect of emodin on AR subcellular localization. The intracellular distribution of AR in LNCaP cells was assessed using immunofluorescence microscopy. AR was mostly localized in cytosol in the absence of androgen for 24 hours (Fig. 2*E*). After stimulation with 1 nmol/L R1881 for 2 hours, AR translocated into the nucleus as clearly indicated by the yellow staining in the nucleus merged from AR (green) and nuclear 4′,6-diamidino-2-phenylindole (red) staining. However, when cells were treated with both R1881 and emodin, AR was

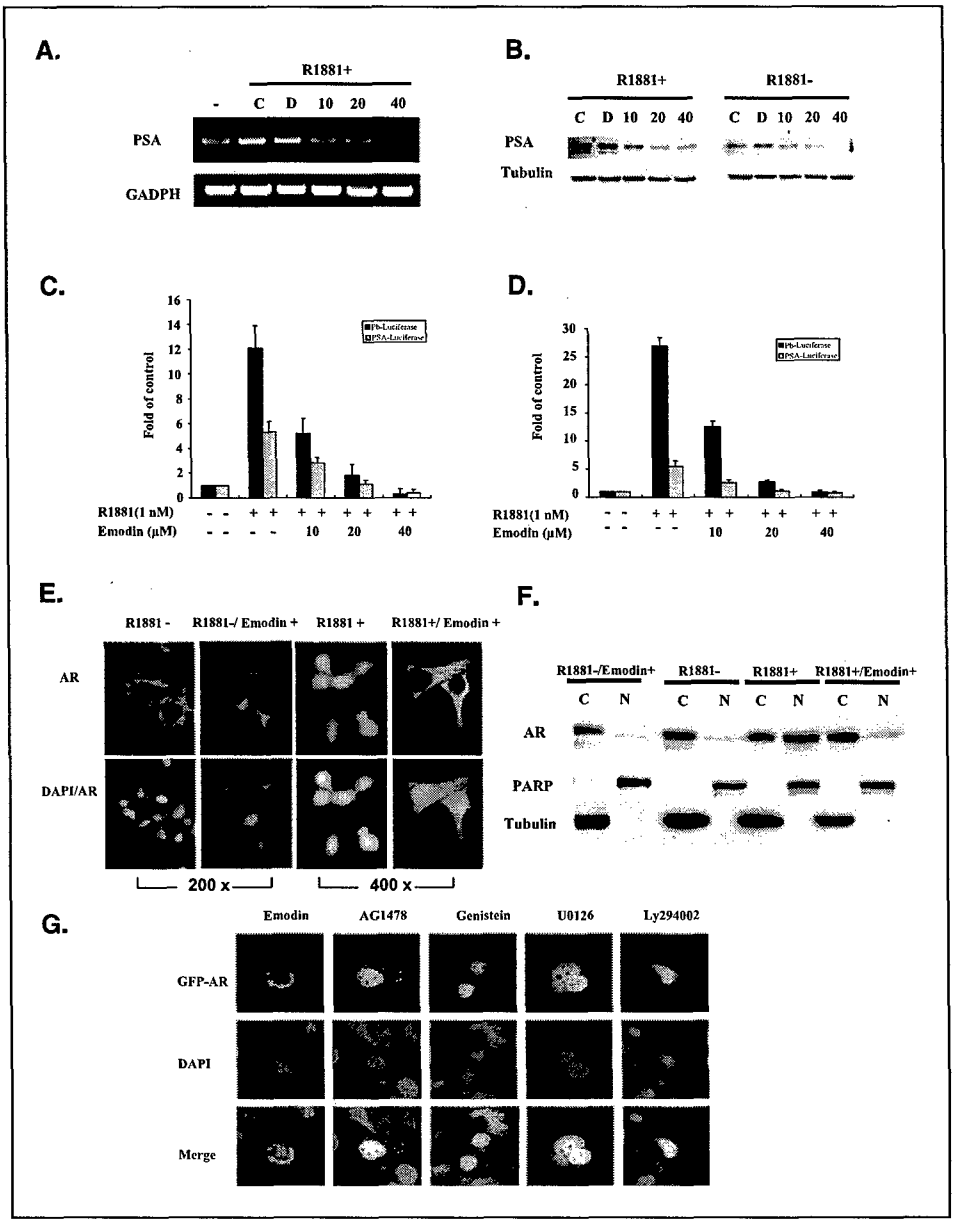

Figure 2. Effects of emodin on AR transcriptional activity and nuclear translocation. *A*, LNCaP cells were treated with solvent [DMSO (*D*)] and various concentrations of emodin (10-40 μmol/L) for 18 hours with (*C*) or without 1 nmol/L R1881. Expression of AR-targeting gene PSA was analyzed by reverse transcription-PCR. Expression of GAPDH was monitored as a control. *B*, PSA protein level was analyzed by Western blotting after emodin treatment and tubulin was measured to ensure consistent loading. *C*, PSA-luc and probasin-luc reporter genes was transiently transfected into LNCaP cells and luciferase activity was measured after emodin treatment. *D*, same as in *C*, except that PC3 cells were cotransfected with pSG5-AR plasmid and reporters. Corresponding β-galactosidase activity was used to normalize luciferase activity. *Columns*, mean of three independent experiments; *bars*, SD. *E*, LNCaP cells were treated with or without 40 μmol/L emodin for 30 minutes and then treated with or without 1 nmol/L R1881 for an additional 2 hours. Cells were fixed in cold methanol and immunostained with FITC-conjugated anti-AR antibody. *F*, results of fractionation experiments on LNCaP cells treated as described in *E*. AR in the cytosol (*C*) and nuclear extracts (*N*) was detected by anti-AR antibody. Anti–poly(ADP-ribose) polymerase (*PARP*) and anti-tubulin antibodies were run to distinguish between nuclear and cytosolic fractions, respectively. *G*, COS-1 cells were transiently transfected with GFP-AR and treated with emodin (40 μmol/L), AG1478 (10 μmol/L), genistein (50 μmol/L), LY294002 (20 μmol/L), or U0126 (20 μmol/L) in the presence of 1 nmol/L R1881 for 2 hours. GFP-AR fusion proteins were detected in living cells using an Axiovert 200 inverted fluorescence microscope. GFP-AR (*green*), 4′, 6-diamidino-2-phenylindole (*DAPI*; *red*), and merging of these two signals (*yellow*).

mostly retained in the cytosol as shown by the AR staining (green) in the cytosol and reduced yellow staining in the nucleus. The emodin-mediated cytosolic retention of AR was also supported by the biochemical approach from cellular fraction experiments (Fig. 2*F*). To further investigate the specific effect of emodin on AR nuclear localization, we did the time lapse experiment by using a fusion protein between AR and GFP to dynamically evaluate AR trafficking in a single living cell. We transfected the AR-GFP fusion protein to COS-1 cells and then tested the effects of different kinds of inhibitors—emodin, AG1478 (a tyrosine kinase inhibitor), genistein (a tyrosine kinase inhibitor), LY294002 (a phosphatidylinositol 3-kinase inhibitor), and U0126 (a mitogen-activated protein kinase inhibitor)—on AR nuclear localization under different times. Before proceeding with this experiment, all the inhibitors were tested and titrated to the optimal dosage for inhibition of their functional targets (data not shown). Under the stimulation by R1881 for 2 hours, only emodin, but not the rest of the inhibitors, prevented AR nuclear translocation (Fig. 2*G*). This effect can be observed for up to 24 hours (data not shown).

Emodin Induces Androgen Receptor Degradation through Proteasome-Mediated Pathway. Because androgen stimulation induces AR nuclear translocation and emodin inhibits this activity resulting in AR accumulation in the cytosol, it becomes very interesting to further investigate the fate of AR under this condition. To this end, we treated LNCaP cells with various concentrations of emodin or for different lengths of time in the presence or absence of 1 nmol/L R1881 and measured AR protein expression. A time- and dose-dependent reduction of the AR protein level was observed (Fig. 3*A* and *B*). Although AR is more stable and has a higher basal level of expression in the presence of ligand, emodin can efficiently deplete AR even in cells undergoing synthetic androgen R1881 stimulation. Thus, emodin-induced depletion of AR is ligand independent. To investigate the molecular

mechanisms for AR depletion, we first investigate whether emodin may have effect on AR mRNA expression. We analyzed AR mRNA level under the treatment with various concentrations of emodin by reverse transcription-PCR and found that emodin did not influence AR mRNA level (Fig. 3*C*). We then further test the effect of emodin on AR protein stability. By using cycloheximide to inhibit protein synthesis, the AR protein stability was significantly reduced under emodin treatment (Fig. 3*D*). To test whether emodin induce AR degradation through the proteasome pathway, treatment with the proteasome inhibitor MG-132 resulted in a marked suppression of emodin-induced AR depletion (Fig. 3*E*). These phenomena were also observed in PC3-AR cells (Fig. 3*F*). Taken together, these findings indicate that emodin induces AR degradation through a proteasome-mediated pathway.

Emodin Disrupts Androgen Receptor-Heat Shock Protein 90 Association and Increases Androgen Receptor-MDM2 Association and Ubiquitination. AR is known to form a heteromeric complex with two molecules of hsp90, which has been shown to participate in regulating the protein stability of ligand-unbound AR. Previous reports showed that some tyrosine kinase inhibitors can reduce the AR protein level (26). We further tested the efficacy of emodin and other tyrosine kinase inhibitors, such as genistein and AG1478, on the reduction of AR and the interaction between AR and hsp90. Due to different drugs having different kinetic and dynamic activities, we chose a relative high dose of AG1478 (10 μmol/L) and genistein (50 μmol/L) to ensure their efficacy (27, 28). Under this situation, emodin-induced reduction of AR is more potent than the other two tyrosine kinase inhibitors especially in the presence of R1881 (Fig. 4*A*). In addition, only emodin can significantly disrupt the association between AR and hsp90 whether treated for a short period (2 hours; Fig. 4*B*) or a longer period (8 hours; data not shown). This result indicates that emodin induces dissociation of AR and hsp90 involving a novel mechanism different from other tyrosine kinase inhibitors. The emodin-induced dissociation between AR and hsp90 may render the incomplete AR heterocomplex more vulnerable to degradation through the proteasome-mediated pathway. It has been shown that AR can be ubiquitinated by an E3 ligase, MDM2 (14). We hypothesized that dissociation of AR and hsp90 by emodin increases the interaction between AR and the E3 ligase MDM2 and therefore enhances degradation. To test this hypothesis, we measured the interaction between AR and MDM2 with or without

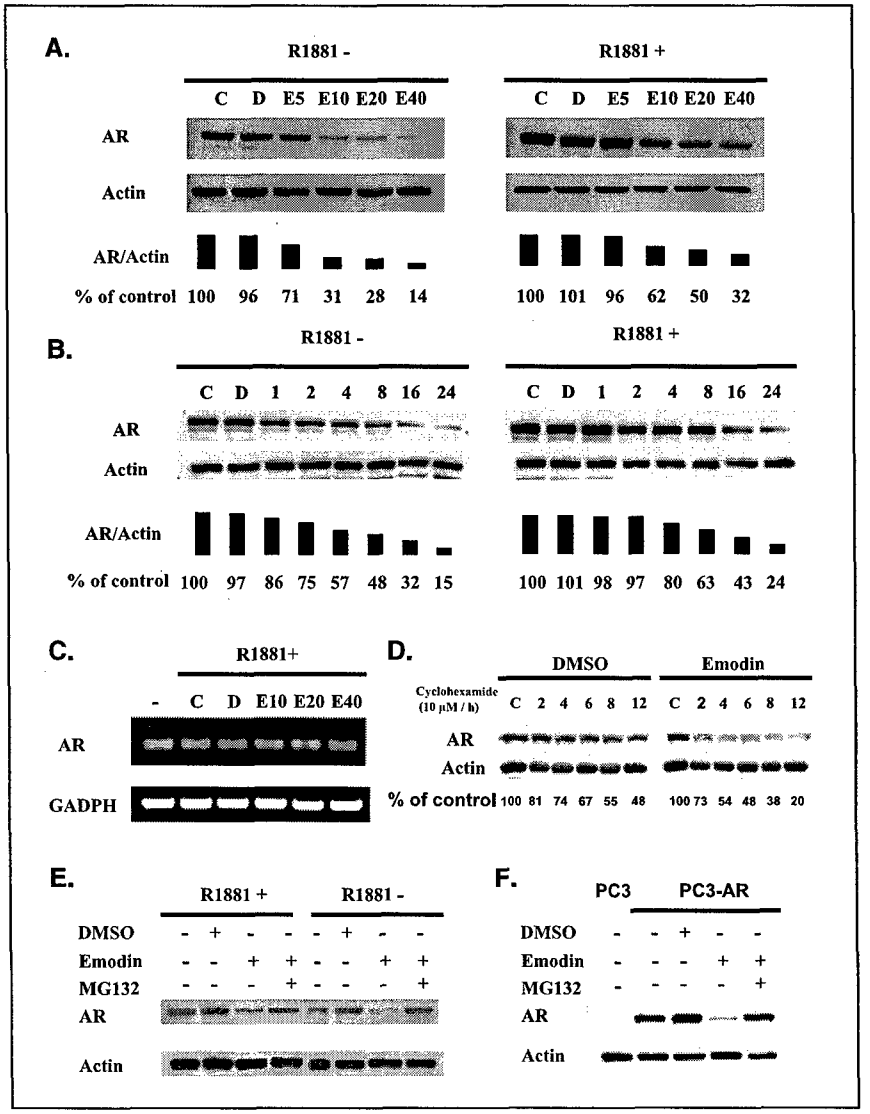

Figure 3. Effect of emodin on AR expression. *A*, LNCaP cells were treated with solvent (DMSO) and various concentrations of emodin [5 (*E5*), 10, 20, or 40 μmol/L] for 18 hours with or without 1 nmol/L R1881. AR protein level was analyzed by Western blot and quantitated by Bio-Rad PDQuest Image software and plotted as the percentage of the control (without emodin) after normalization with actin. *B*, LNCaP cells were treated with 40 μmol/L emodin for various lengths of time. AR protein level was measured by immunoblotting. *C*, expression of the AR gene in LNCaP cells was analyzed by reverse transcription-PCR after treatment with various concentrations of emodin for 18 hours. Expression of GAPDH was monitored as a control. *D*, LNCaP cells were treated with 40 μmol/L emodin and 10 μmol/L cycloheximide for various lengths of time. DMSO was added to the control. AR protein level was measured by Western blot analysis. *E*, LNCaP cells were treated with 40 μmol/L emodin and 5 μmol/L MG-132 with or without R1881 for 12 hours. DMSO was added to the control. AR protein level was measured by Western blot analysis. *F*, PC3-AR cells were treated with 40 μmol/L emodin and 5 μmol/L MG-132 for 12 hours. DMSO was added to the control. AR protein level was measured by Western blot analysis.

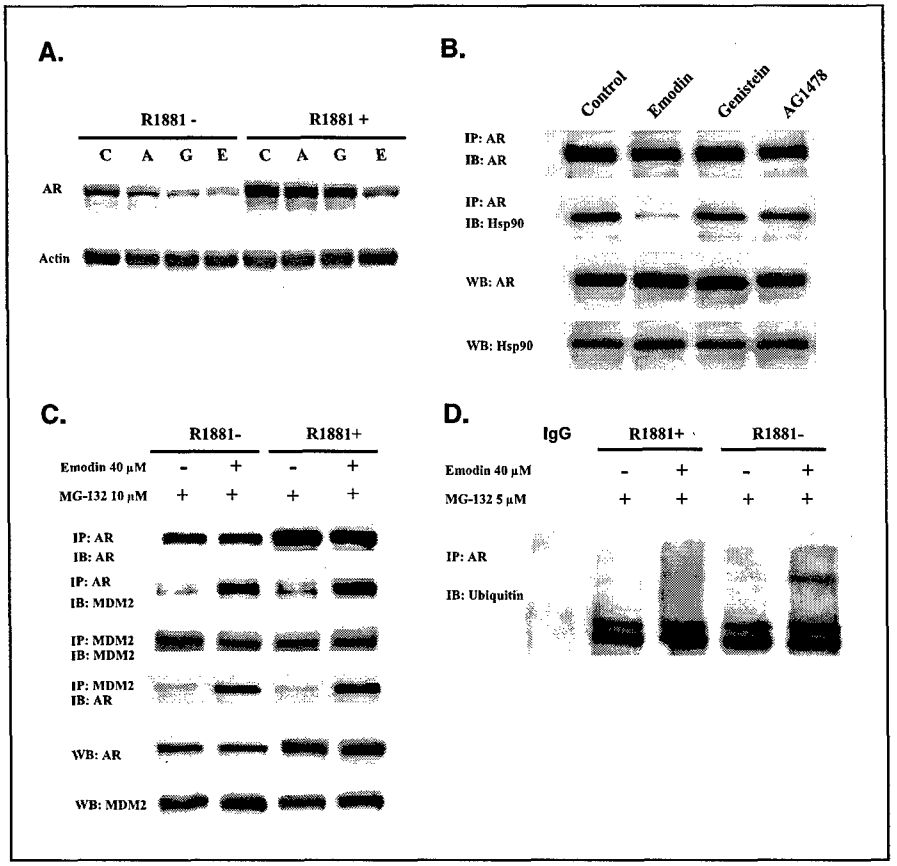

Figure 4. Effect of emodin on the associations between AR and hsp90 and AR and MDM2. *A*, LNCaP cells were treated with emodin (40 μmol/L), AG1478 (10 μmol/L), and genistein (50 μmol/L) with or without R1881 for 18 hours. AR protein level was measured by Western blot analysis. *B*, LNCaP cells were incubated with c-FBS medium overnight and then treated with or without emodin (40 μmol/L), AG1478 (10 μmol/L), and genistein (50 μmol/L) for 2 hours. Immunoprecipitation and Western blot analysis were done with indicated antibodies. *WB*, Western blotting of whole cell lysates. *C*, LNCaP cells were incubated in c-FBS medium in the presence or absence of 1 nmol/L R1881 overnight and then treated with or without emodin (40 μmol/L) in the presence of MG-132 (10 μmol/L) for 2 hours. AR immunoprecipitation was done as in *B*, except that immunoblotting was done with anti-MDM2 antibody rather than anti-hsp90 antibody. *D*, LNCaP cells were incubated in c-FBS medium overnight and then treated with or without emodin (40 μmol/L) in the presence of MG-132 (5 μmol/L) and with or without 1 nmol/L R1881 stimulation for 8 hours. AR immunoprecipitation was done and immunoblotting was done with anti-ubiquitin antibody.

emodin treatment in LNCaP cells. Emodin treatment increased the association between the endogenous MDM2 and AR as evident from coimmunoprecipitation experiments using antibodies against MDM2 and AR (Fig. 4*C*). The emodin-induced AR-MDM2 association was also shown in PC3-AR cell line (data not shown), suggesting that this is a general phenomenon in different cell types. In addition, emodin treatment resulted in an increase of AR ubiquitination (Fig. 4*D*). Taken together, these results indicate that emodin dissociates hsp90 from AR and enhances AR and MDM2 association, which may lead to further ubiquitination and degradation.

Emodin Inhibits PC3-Androgen Receptor Tumor Growth and Down-regulates Androgen Receptor and Prolongs Survival of C3(1)/SV40 Transgenic Mice. The above results established a novel molecular mechanism to explain how emodin may down-regulate AR and inhibit prostate cancer cell growth in an *in vitro* cell culture system. To further investigate the *in vivo* antitumor activity of emodin, first we chose PC3 and PC3-AR xenografts as animal models. Low-dose (40 mg/kg) emodin showed significant antitumor activity in mice bearing PC3-AR xenograft ($P < 0.01$); however, the inhibitory effect was not effective in PC3 xenograft (Fig. 5*A* and *B*). Furthermore, we used C3(1)/SV40 transgenic mice as a second *in vivo* experimental model. It is known that the male mice of this strain will develop AR-positive prostate cancer and eventually die with prostate cancer because the SV40 large T antigen was driven by the promoter of rat prostatic steroid binding protein [C3(1)] gene in the transgenic mice (29). Because the carcinogenesis of C3(1)/SV40 transgenic mouse model is primarily driven by AR, this transgenic mouse model provides a clean background, and by using the tumor development as a readout, we can more specifically test the effect of emodin on AR *in vivo*. We treated male C3(1)/SV40 transgenic mice with either emodin (40 mg/kg) or DMSO i.p. every other day while the mice were 4 weeks old and with no signs of tumors. Emodin-treated mice have significantly longer survival than the control group ($P < 0.001$; Fig. 5*C*). Emodin-treated mice maintained body weight gain; in contrast, DMSO-treated control mice significantly lost body weight gain after age 20 weeks ($P < 0.05$; Fig. 5*D*). We noticed that not only the size but also the hair grooming and cage activity were clearly different between emodin-treated and control groups. In general, emodin-treated mice looked much healthier, but the control mice seemed to be in distress with labored breathing, cachectic and lethargic patterns (Fig. 5*E*). This result showed that emodin not only has low drug toxicity but also maintains the physical activity of C3(1)/SV40 transgenic mice by preventing tumor progression. To further address whether the biological effect of emodin is related to its ability to down-regulate AR, we analyzed AR expression of tumor tissues from both emodin-treated and control groups. Immunohistochemical staining using an AR-specific antibody clearly indicated that prostate cancer tumor tissues from emodin-treated mice were much weaker than those from the control group (Fig. 5*F* and *G*). The same results were also obtained by using Western blot analysis of fresh tumor samples taken from other pairs of mice (Fig. 5*H*). In addition, when we analyzed the tumor progressive status by histopathologic investigation of mice at age 21 weeks, the emodin-treated mice clearly had a lower incidence of tumor invasion to the periurethral muscle structure (1/7) compared with the control group (7/7; Fig. 5*I*). Our results showed that emodin indeed can down-regulate AR in C3(1)/SV40 transgenic mice model. Considering the success of the recent finasteride prevention trial for prostate cancer and the dilemma it

created due to its androgen deprivation nature, emodin, which directly down-regulates AR in a ligand-independent manner, may have an advantage for further development as a therapeutic and chemopreventive agent for prostate cancer.

Discussion

Emodin, an active extract of *R. palmatum*, has been shown to have multiple biological activities, including anti-inflammatory, antibacterial, diuretic, immunosuppressive, vasorelaxant, and anticancer effects (30–33). We have shown previously that emodin inhibits HER-2/*neu* tyrosine kinase activity, and it preferentially suppresses growth and induces differentiation of the HER-2/*neu*-overexpressing breast cancer cells but has no effects on normal cells (34). Here, we found a novel emodin-mediated mechanism of inhibition of prostate cancer cell growth, especially AR-positive cells, through down-regulation of AR. AR mediates growth-promoting and survival effects through either genotropic action by transcriptional activation of target genes and nongenotropic action by activation of phosphatidylinositol 3-kinase/Akt, mitogen-activated protein kinase, and protein kinase C pathways (35–37). In PC3 cells stably expressing AR, androgens are able to activate these nongenotropic pathways (35, 37). Our results showed that both LNCaP cells with endogenous AR and PC3-AR stable transfectants are more sensitive to low-dose emodin. Emodin-mediated down-regulation of AR may inhibit both genotropic and nongenotropic pathways and make cells more vulnerable and result in growth inhibition.

AR is a nuclear transcription factor. Nuclear translocation is a key step for AR in response to androgen stimulation (38), and this process initiates the transactivation of downstream target genes to promote prostate cancer cell proliferation and survival. Our results showed that emodin inhibits androgen-mediated AR nuclear translocation and induces dissociation of AR and hsp90. However, this phenomenon did not occur with treatment of other kinase inhibitors, including receptor tyrosine kinase, mitogen-activated protein kinase, and Akt pathway inhibitors. Consistent with

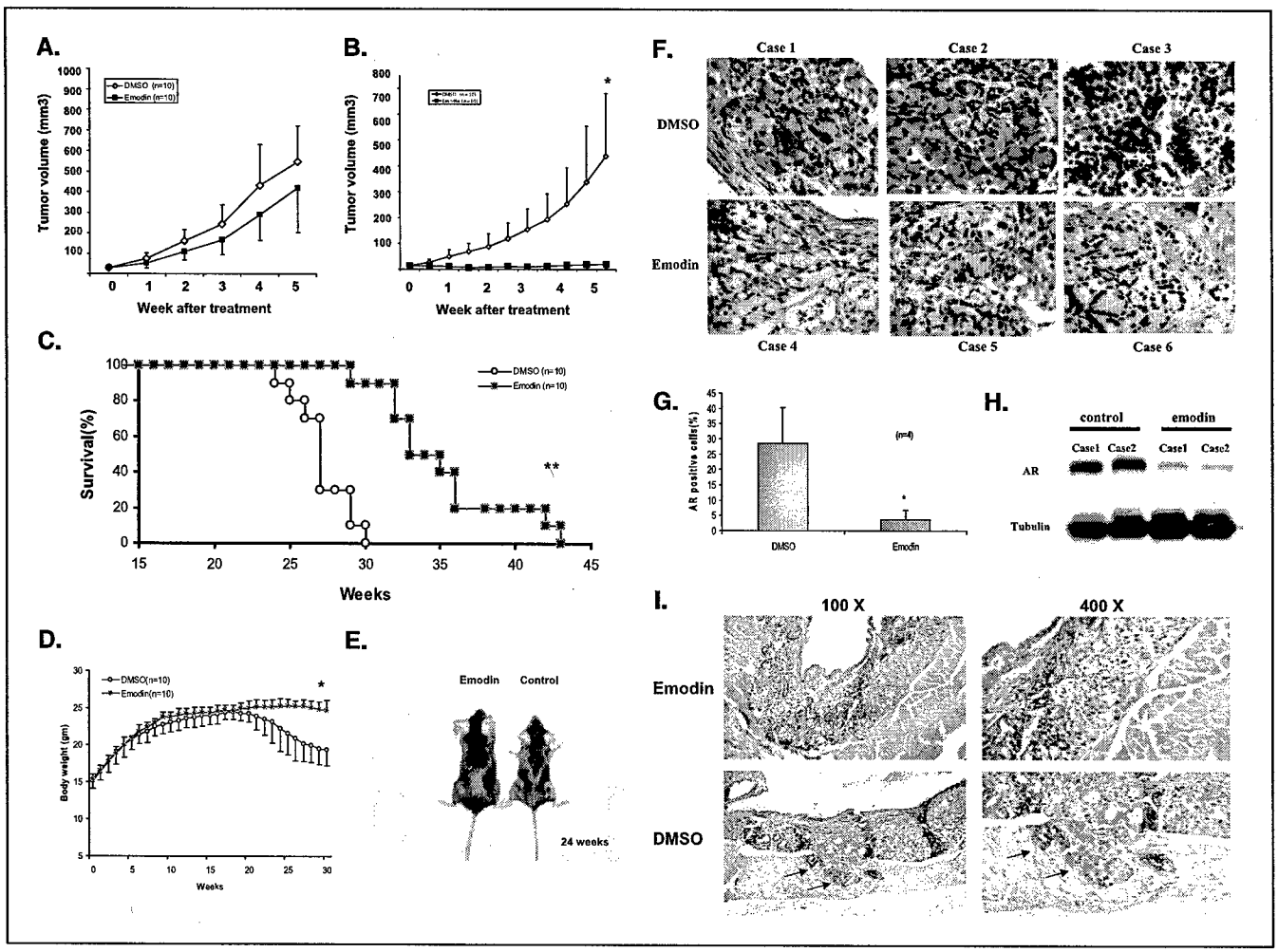

Figure 5. Effect of emodin on *in vivo* animal models. *A* and *B*, *in vivo* antitumor activity of emodin on nude mice bearing PC3 and PC3-AR xenografts. *, $P < 0.01$. *C*, 4-week-old mice were i.p. injected with 40 mg/kg emodin or DMSO every other day. Survival of mice treated with emodin or DMSO. **, $P < 0.001$. *D*, body weight gain profiles of emodin-treated and control transgenic mice from 0 to 30 weeks. *, $P < 0.05$. *E*, emodin-treated mouse maintained body weight gain and physical activity not seen in the control mouse (24 weeks old). *F*, tumor tissue sections from 21-week-old emodin-treated and control mice with identical treatment as survival experiment in *C* were stained with antibody specific to AR. *G*, quantitative analysis of AR expression between emodin-treated and control mice were assessed in 2,500 cells in several different views. *, $P < 0.05$. *H*, Western blot analysis of fresh tumor tissues obtained from two sets of 21-week-old emodin-treated and control mice with identical treatment as survival experiment in *C*. *I*, H&E-stained tumor tissue sections from the emodin-treated and control mice. Low magnification, ×100; high magnification, ×400. Control mice showed tumor invasion of periurethral muscle (*black arrow*) not seen in emodin-treated mice.

previous reports, the association between AR and hsp90 is not disrupted by other tyrosine kinase inhibitors (39). These results suggest that emodin-mediated inhibition of AR nuclear translocation is specific and involves a novel mechanism correlating with the dissociation of AR and hsp90. It has been shown that hsp90 function is required for AR nuclear translocation, which is consistent with our observation (40).

The steroid receptors interact with hsp90 and other co-chaperones to create a mature conformation for proper protein function (40–42). Without hsp90 binding, the misfolded or unfolded proteins will be recognized and degraded by the ubiquitin-proteasome system (43). Emodin treatment induces the dissociation between AR and hsp90 and increases the association of AR and MDM2, providing a plausible mechanism for the involvement of MDM2 as an E3 ligase for the emodin-mediated AR degradation. Previous studies have shown that the hsp90 inhibitors, such as geldanamycin and its derivatives, can directly bind to the ATP-binding pocket of hsp90 and inhibit its function and then further induce steroid receptor degradation (39, 41, 44–46). Although both emodin and geldanamycin share the similar feature of abrogating the interaction between AR and hsp90, their mechanisms are different. Emodin induces dissociation of AR and hsp90, but geldanamycin cannot. It will be interesting to see whether MDM2 is also responsible for the geldanamycin-induced AR degradation.

A recent study showed that overexpression of AR in hormone-refractory xenograft model is consistent with observations in human clinical specimens, and overexpression of AR promotes the transition of hormone-dependent xenograft into a hormone-independent xenograft (47, 48). These observations indicate that reducing AR expression to a critical level would contribute to preventing prostate cancer progression. Emodin-induced degradation of AR occurs in a ligand-independent manner. Thus, as long as AR is functional in prostate cancer regardless of androgen-dependent or androgen-independent status, emodin should inhibit cancer cell growth because of the induction of AR degradation. In addition to enhanced AR expression, other proposed mechanisms include cross-talk between AR and other signal transduction pathways also involved in development of the hormone-refractory state. Overexpression of receptor tyrosine kinases, such as HER-2/*neu*, also is known to contribute to prostate cancer development (49). In this regard, it should be mentioned that high-dose emodin also associates with the activity to inhibit tyrosine kinase and suppress HER-2/*neu*-mediated tumorigenecity in breast cancer cells (30, 34). The dual functions of anti-AR and anti–tyrosine kinase may be an advantage for using emodin as a chemopreventive agent to avoid the development of aggressive prostate cancer phenotype.

In addition to the observation of the effects of emodin in the *in vitro* cell culture system, the *in vivo* effects were also observed in emodin-treated C3(1)/SV40 transgenic mice. Our results showed that emodin induced degradation of AR in the tumor tissues, suppressed tumor development, and prolonged animal survival. Because the carcinogenesis of C3(1)/SV40 transgenic mice model is primarily driven by AR, emodin-induced degradation of AR may compromise the SV40 oncogene expression, which may not reflect the real chemopreventive effect on inhibition of tumor development in C3(1)/SV40 transgenic mice. However, AR is responsible for the initiation and progression of prostate cancer in a real situation. Our transgenic model showed that emodin efficiently down-regulated AR *in vivo*, which suggests that emodin have the potential as a chemopreventive agent for prostate cancer. Emodin-treated mice maintained their body weight gain and physical activity, suggesting that the effective dose of emodin, which suppresses tumor progression, is well tolerated and nontoxic. Tumor invasion resulting in distant metastases is the major cause of prostate cancer–related death (50). Emodin-treated transgenic mice had lower incidence of periurethral invasion, which represents the preventive effect of emodin contributing to prolong the survival of transgenic mice.

Considering the dilemma created by the recent Prostate Cancer Prevention Trial, this current study provides the evidence to support that directly targeting AR rather than its ligands could be a good strategy in the treatment or prevention of prostate cancer. Emodin may have the potential as a novel anti-AR therapeutic and preventive agent for prostate cancer.

Acknowledgments

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Title/Referral Page
No Page Limit

a. Proposal title (up to 160 characters)

TARGETED GENE THERAPY FOR ANDROGEN DEPENDENT AND INDEPENDENT PROSTATE CANCER

b. Proposal log number

PC051055

c. PI's full name (first, middle initial, last)

MIEN-CHIE HUNG

d. PI's institution

THE UNIVERSITY OF TEXAS M. D. ANDERSON CANCER CENTER

e. Award mechanism

IDEA DEVELOPMENT AWARD

f. Please indicate if this is a:

☒ NEW proposal
☐ DUPLICATE proposal to another FY05 CDMRP program
☐ RESUBMITTED proposal to this program

g. Keyword descriptive technical terms

PROSTATE CANCER, GENE THERAPY, ANIMAL MODELS

h. Conflicts of interest: Include the following information (no page limit)

Name	Institutional Affiliation(s)	Role(s) on Proposed Project or Perceived Conflicts of Interest
MIEN-CHIE HUNG, PH.D.	**UTMDACC**	**PRINCIPAL INVESTIGATOR**
XIAOMING XIE, PH.D.	**UTMDACC**	**CO-INVESTIGATOR**
TAI-LUNG CHA, M. D.	**UTMDACC**	**RESEARCH ASSISTANT**

FY05 PCRP Idea Development Award Proposal
Table of Contents/Checklist

Proposal Log Number: PC051055

Principal Investigator: Hung, Mien-Chie
Last Name *First Name* *MI*

Proposal Title: Targeted Gene Therapy for Androgen Dependent and Independent Prostate Cancer

NOTE: Exceeding page limits may result in proposal rejection prior to peer review. Submit only materials specifically requested or required in this program announcement. Submission of additional materials may be construed as an attempt to gain an unfair advantage.

Proposal Relevance Statement

For advanced or metastatic prostate cancer that is initially androgen-dependent (ADPC) hormonal deprivation therapy (ADT) is the primary choice. However, this modality eventually fails because prostate cancer frequently develops to an androgen-independent state within 2 years. Androgen-independent prostate cancer (AIPC) is an untreatable form of prostate cancer. In the prostate cancer gene therapy setting, the most frequently reported prostate specific promoters, such as PSA, PB, and hK2, provide a possibility of targeting transgene expression to the AR^+ prostate cancer (and normal) cells under the condition of androgen stimulation. However, this type of promoter may not be useful for the treatment of AIPC or AR^- prostate cancer because activation of these promoters is required for the androgen-AR complex to bind the androgen response element.

Recently however, a series of innovative integrated tactics for gene therapy of ADPC and AIPC has been developed in our laboratory: (1) using the base of the hTERT promoter (hTERTp) fused with the TSTA system and WPRE as well as ARR2, we engineered the ATTP promoter composite (ARR2PB.hTETp.TSTA-WPRE), which targets the transgene expression to the ADPC and AIPC, AR^+ and AR^- prostate cancer. (2) We developed a Bik mutant (BikDD) gene that possesses potent anti-tumor activity in prostate cancer cells *in vitro* and *in vivo*. (3) We have improved the non-viral delivery system obtaining a stabilized polymer-liposome compound, SN, which forms a 70-170-nm nanopaticle with plasmid DNA and is a more effective systemic delivery system for gene therapy. And (4) we have established an *in vivo* and real-time imaging system for monitoring of tumor growth and metastasis of prostate cancer.

In this proposal, three tasks will be carried out to accomplish our goals. In task 1, we will test the targeted antitumor activity of ATTP-BikDD in ADPC and AIPC *in vitro* and in EZC (easy see using the IVIS™ system)-prostate cancer orthotopic models. Prostate cancer cell lines that stably express firefly luciferase will be generated and EZC-prostate cancer orthotopic models will be created. EZC-prostate cancer orthotopic mice will be systemically administrated by i.v. injection of ATTP-BikDD plasmid DNA/SN complexes. The *in vivo* antitumor activity of ATTP-BikDD and expression profiling of BikDD will be evaluated using the IVIS™ living imaging system and traditional methods, as well as after castration. For task 2, we will test the targeted antitumor activity of ATTP-BikDD in an EZC-TRAMP model. We will crossbreed EZC-prostate mice with TRAMP mice, to create bigenic mice, EZC-TRAMP, in which the tumors should glow. The antitumor activity of ATTP-BikDD will be further tested in the EZC-TRAMP model. In task 3, we will evaluate the therapeutic efficiency of gene therapy with combined conventional chemotherapy. Since non-viral methods of gene transfer are less immunogenic than adenoviral vectors and generally considered a "safer" delivery vehicle for gene therapy, we will employ our recently developed non-viral delivery system (SN) to systemically deliver the ATTP-BikDD plasmid DNA into the tumor cells. A non-invasive imaging system, IVIS™ xenogen system, will allow us to monitor tumor growth in real-time *in vivo*, which is essential for preclinical and clinical studies.

The successful targeted expression of ATTP-driven BikDD in AR^+ and AR^- prostate cancer cells *in vitro* and *in vivo* will provide a foundation for the eradication of ADPC and AIPC. The glowing tumor models of EZC-prostate cancer orthotopic and EZC-TRAMP mice will pave an avenue for real-time non-invasive monitoring of the kinetics of tumor growth and metastasis. The ultimate achievement of the ATTP-BikDD/SN strategy would lead to an innovative treatment for men with advance prostate cancer, regardless of ADPC and AIPC.

1. BACKGROUND AND PRELIMINARY RESULTS

1.1. A strong prostate cancer-specific promoter for gene therapy of Androgen-dependent (ADPC) and androgen-independent prostate cancer (AIPC) is needed. For patients with advanced or metastatic prostate cancer that is initially androgen-dependent (ADPC), hormonal deprivation therapy (ADT) is the primary choice. However, this modality eventually fails because prostate cancer frequently develops to an androgen-independent state within 2 years [1, 2]. Androgen-independent prostate cancer (AIPC) is an untreatable form of prostate cancer in which the normal dependence on androgen for growth and survival has been bypassed [3]. AIPC is a lethal form of prostate cancer that progresses and metastasizes. In the prostate cancer gene therapy setting, prostate specific promoters, such as prostate-specific antigen (PSA) [4-6] and probasin (PB) [7-10](Appendix 1), have been recently developed and tested by many groups including us. The co-PI, Dr. Xie, while working with Dr. David Spencer at Baylor College of medicine, has recently developed a human kallikrein 2 (hK2)-based promoter, hK2-E3/P, and demonstrated that the activity of hK2-E3/P promoters is comparable to that of the cytomegalovirus (CMV) promoter [11](Appendix 2), which provides a possibility of targeting transgene expression to AR^+ tissues including normal prostate epithelia cells, androgen-dependent (ADPC) or AR^+ (androgen receptor) prostate cancer cells under the condition of androgen stimulation. However, this type of promoter may not be useful for the treatment of AIPC or AR^- prostate cancer because the activation of these promoters is required for the binding of the androgen-AR complex to the androgen response element (ARE). Currently, several prostate-specific promoters functioning in AR^- prostate cancer or in androgen-independent manner have been reported by multiple laboratories including 1) the prostate-specific membrane antigen (PSMA) enhancer can increase promoter activity in PSMA-expressing LNCaP and LAPC-4 cells. However, it exhibits very low activities in the androgen-independent cell lines PC-3 and DU145 [12]; 2) the rPB-DR (6) promoter, by substituting RARE (retinoic acid-response element) for the two AREs of PB, is active in both AR^+ (LNCaP,) and AR^- (PC-3 and DU145) cells, but its activity is 10-fold lower than that of the SV40 promoter [13]; and 3) the PPT promoter, by combining PSA and PSMA enhancers with TART promoter (T cell receptor γ-chain alternate reading frame protein), also shows less activity than the CMV promoter, particularly in PC-3 and DU145 cells [14]. **Thus, the reported androgen-independent promoters exhibit much lower activity in ADPC and AIPC than the CMV promoter. Therefore, developing a strong promoter that is active in both ADPC and AIPC (or AR^+ and AR^-) prostate cancer with specificity, is a big challenge for efficient gene therapy of prostate cancer.**

1-2. A robust and specific promoter for gene therapy of ADPC and AIPC is currently developed in our lab.
1-2-1. TSTA and WPRE enhance the activity of the hTERT promoter. We have recently developed a robust prostate cancer promoter composite using the hTERTp promoter as a base in combination of TSTA and WPRE (please see below). Telomerase is an RNA-dependent DNA polymerase that synthesizes telomeric DNA. The telomerase holoenzyme is composed minimally of a constitutively expressed, template-containing RNA subunit and a catalytic protein subunit (human telomerase reverse transcriptase, hTERT)[15]. Telomerase expression is highly elevated in malignant tumors and cancer cell lines uncluding prostate cancer cells but not in normal tissue [16]. Recently, the hTERT promoter (hTERTp) has been used for cancer gene therapy [17-19]. However, the activity of this unmodified hTERTp is much weaker than that of the commonly used CMV promoter [17, 20, 21]. To augment the transcriptional activity of hTERTp, the two-step transcriptional amplification (TSTA) system [22, 23] should be a good choice. In this system, the first step involves the tissue-specific expression of the GAL4-VP16 fusion protein. In the second step, GAL4-VP16, in turn, drives target gene expression under the control of GAL4 response elements in a minimal promoter. The use of TSTA can lead to amplified levels of transgene expression. In addition, the post-transcriptional regulatory element of the woodchuck hepatitis virus (WPRE) involves modification of RNA poly(A), RNA export, and/or RNA translation [24]. WPRE can stimulate 2-to 7-fold more luciferase expression *in vitro* and 2- to 50-fold *in vivo* [25, 26]. Furthermore, long-term transgene expression can be mediated by WPRE-containing adenoviral vectors [27]. Therefore, we used TSTA and WPRE to amplify the hTERTp activity (Fig. 1A). The hTERTp activity is increased in both LNCaP and PC-

3 cells through the TSTA system, and is further enhanced by WPRE (Fig. 1B). In combination with TSTA and WPRE, **the activity of hTERTp-TSTA-WPRE is comparable to or even 1.5-fold greater than that of the CMV promoter, in PC-3 and LNCaP cells, respectively. Importantly, its activity remains silent in human normal lung fibroblast cells WI-38 and in normal tissue of the mouse model further confirmed later (Section 1-2-3.).**

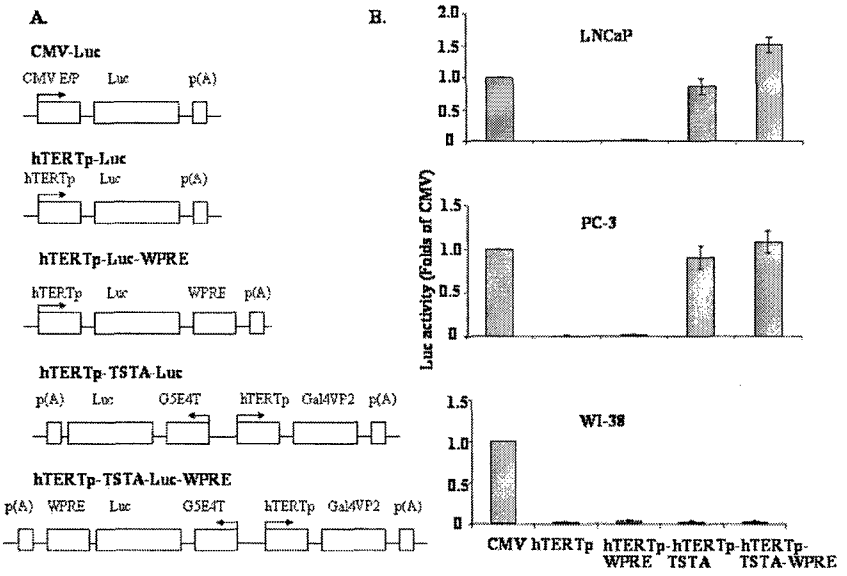

Fig. 1. Comparison of firefly luciferase activity under the control of CMV promoter and hTERTp-based promoter composites. (A) A schematic diagram of reporter constructs. (B) Prostate cancer LNCaP and PC-3, and normal human fibroblast WI-38 cells lines were transiently co-transfected with reporter plasmid DNA and the internal control vector pRL-TK. 48 h later, the dual luciferase ratio was measured. Shown are the luciferase activities (folds) in relative to the CMV promoter (setting at 1).

1-2-2. The *cis*-acting ARR2 element further boosts the activity of TSTA- and WPRE- modified hTERTp in response to androgen stimulation *in vivo*. In most cases of recurrent or metastatic prostate cancer through ADPC to AIPC, the AR gene is amplified and/or AR is overexpressed and still able to bind to androgen (or androgen analog) following by binding to the androgen responsive element (ARE), resulting in transcriptional activation [28, 29]. In this regard, the therapeutic index should be greatly improved if the promoter contains an ARE, which binds to the androgen (or androgen analog)/AR complexes, leading to stimulation of the therapeutic gene expression. To accomplish this goal, the ARR2 element (androgen-receptor responsive element 2), which is derived from ARR2PB[8, 9], was fused to upstream of hTERTp in our newly constructed systems, to generate

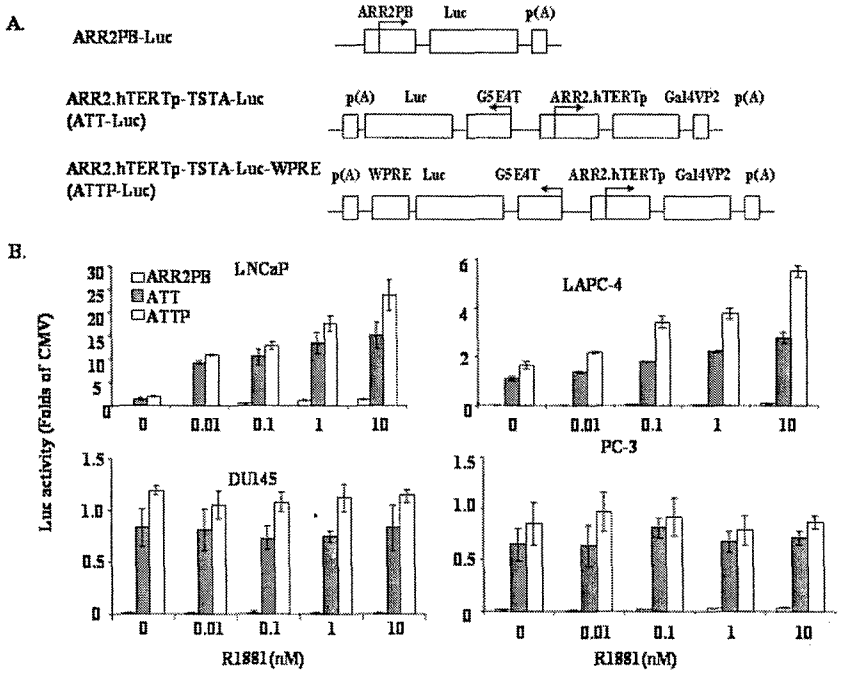

Fig. 2. *Cis*-acting ARR2 element further boosts TSTA- and/or WPRE modified hTERTp in response to androgen stimulation. (A) Schematic diagram of reporter constructs. (B) Cells were transiently co-transfected with reporter plasmid DNA and the internal control vector pRL-TK. 48 h after incubation with R1881, the dual luciferase ratio was measured. Shown are the luciferase activities (folds) in relative to that of the CMV promoter (setting at 1)

ATT-Luc (pGl3-ARR2.hTERTp-TSTA-Luc) and **ATTP-Luc (pGl3-ARR2.hTERTp-TSTA-Luc-WPRE)** (Fig. 2A). ARR2PB-Luc (pGl3-ARR2PB-Luc) was used as a control and CMV-Luc as a reference tool. The cells were transiently co-transfected with the constructs and the internal control vector pRL-TK and incubated with increasing concentrations of androgen analog, R1881., Indeed, **the activities of ATT and ATTP composites were increased in an androgen-dependent manner, up to 15- and 25-fold greater in AR^+ADPC LNCaP cells and up to 2.8- and 5.5-fold in AR^+AIPC LAPC-4 cells, respectively, than that of the CMV promoter (Fig. 2B).** ARR2 does not interfere with the transcriptional activities of hTERTp-TSTA and hTERTp-TSTA-WPRE in PC-3 and LNCaP cells (Fig. 1B and Fig. 2B), and their specificity in normal cells (data not shown). Compared with ATTP and ATT, ARR2PB is much less active in AR^+ (LNCaP and LAPC-4) and almost inactive in AR^- (PC-3 and DU145) cells (Fig.2B). **Thus, our newly generated ATTP and ATT are highly active in all four cell lines tested, which activities are comparable in AR^- AIPC (PC-3 and DU145) cells to, and much stronger in AR^+ ADPC (LNCaP) and AR^+ AIPC(LAPC-4) cells than that of the CMV promoter, and importantly remain silent in the normal cells.**

1-2-3. ATTP is robust in ADPC and AIPC xenografts *in vivo*. To further determine whether the activity and specificity of ATTP would be maintained *in vivo*, we established male BABL/c nu/nu mouse models of s.c. LNCaP and PC-3 xenografts. Mice bearing LNCaP or PC-3 tumors were i.v. injected with 50 μg of pGL3-ATTP-Luc and pGL3-CMV-Luc in DNA:liposome complexes, once a day for three consecutive days. Mice-bearing LNCaP tumors were *in vivo* imaged with a non-invasive imaging system [30] for two minutes every day and sacrificed 24 h after the last injection. Bioluminescent imaging showed very brilliant light spots in the area of the thorax (lung/heart) of mice treated with CMV-Luc, but **almost none in the same area of mice treated with ATTP-Luc (Fig.3A).** To more unambiguously determine the source of the light, we sacrificed the mice immediately after live imaging, dissected their major organs to be imaged *ex vivo*. We verified that the strongest photo-emitting organ was the lung of mice treated with CMV-Luc, whereas **the signal from the lungs of mice treated with ATTP-Luc was undetectable (Fig. 3B).** To

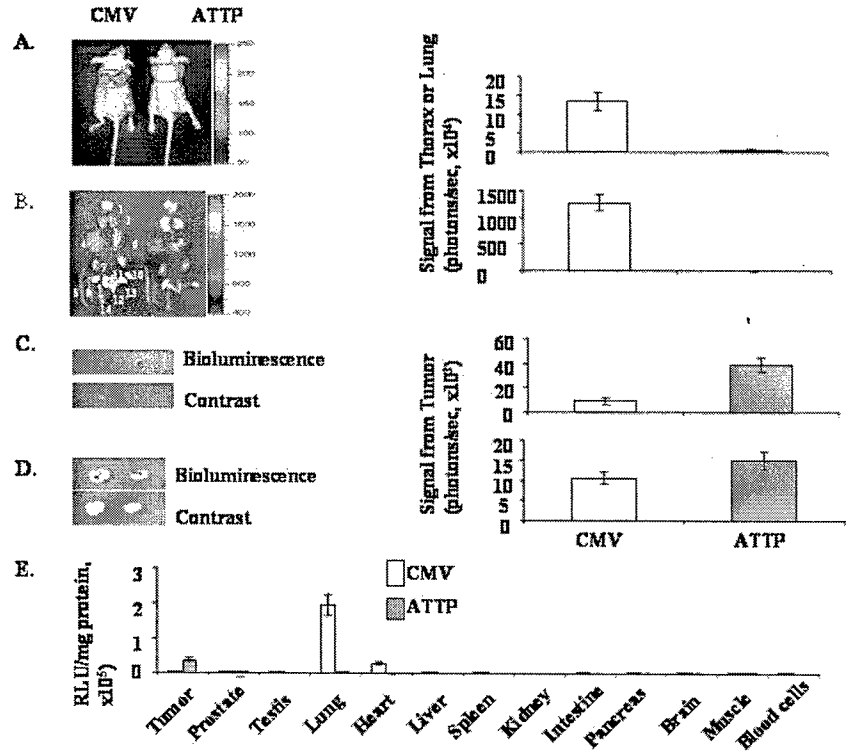

Fig. 3. *In vivo* luciferase expression in LNCaP and PC-3 xenografts after systemic delivery of plasmid DNA. Male nude mice bearing s.c LNCaP tumors were i.v. injected with DNA (CMV-Luc or ATTP-Luc):liposome complexes. Mice were anesthetized, and imaged for 2 min with an IVIS™ imaging system following an i.p. injection of D-luciferin. The imaging shown is 24 h after the last injection. (A). Mice were then immediately dissected and imaged ex vivo for 2 min (B). The dissected tumors were then immediately imaged for 10 min (C). (D). *Ex vivo* PC-3 tumors were imaged (as C) after systemic delivery of CMV-Luc or ATTP-Luc plasmid DNA to the PC-3 tumor-bearing mice. The quantitative signal was presented (right). (E). *In vivo* biodistribution of luciferase expression in the LNCaP tumor model. Tissue specimens from tumor and organs as shown were dissected and measured for luciferase activity with a luminometer.

increase signal strength by prolonging photon-exciting time, the dissected tumors were then immediately imaged for 10 min. The tumors from mice treated with ATTP-Luc produced much stronger signal than ones from mice treated with CMV-Luc (P, 0.002) (Fig. 3C). Consistent with the *in vivo* and *ex vivo* imaging results, the luciferase activities from lungs and hearts of mice treated with CMV-Luc were significantly greater than that of mice treated with ATTP-Luc. In contrast, **the luciferase activity from tumors of mice treated with ATTP-Luc was 14.5-fold greater than that of the tumors of mice with CMV-Luc (P, 0.004) (Fig. 3E). The cancer-specific index (the luciferase activity of tumors to lung) [31] was 14.3 for ATTP-Luc in contrast to 0.012 for CMV-Luc in the LNCaP tumor model.** Due to the fact that PC-3 cells are AR^- the signal from PC-3 tumors treated with ATTP-Luc was not as great as that from LNCaP tumors. However, the signal from **the PC-3 tumors of mice treated with ATTP-Luc was still stronger than that of mice treated with CMV-Luc (Fig. 3D) and the cancer-specific index (3.9) of ATTP-Luc is still much better that that (0.015) of CMV-Luc** (data not shown). **Taken together, the "chimeric" ATTP is able to direct a gene of interest to the prostate tumor (both AR^+ and AR^-) at least as efficiently as that of the CMV promoter in the AR^- prostate cancer, and much more efficiently in AR^+ prostate cancer whereas there is almost no expression in normal cells. The broad prostate cancer specificity and a high transcriptional activity of ATTP will be further investigated in the current proposal.**

1-3: SN is an effective systemic delivery system for gene therapy. In an attempt to establish a non-viral gene delivery system that can target metastatic cancers by systemic delivery of a therapeutic gene, we have recently developed a stabilized non-viral liposome (SN). SN is a polymer-coated cationic liposome formulation composedof1,2-dipalmitoyl-sn-glycerol-3-ethylphosphocholine,1,2-dipalmitoyl-sn-glycero-3-phosphoethanoa-mine-N-polyethyleneglycol-5000 (DSPE-PEG) and polyethylenimine. [32]. The DNA is entrapped in the liposome after the thin-lipid film is hydrated and extruded through a filter with 0.2 μm diameter pores [32]. The lipid/DNA ratio is 12:1, and the size of liposomal DNA particle is 70-170 nm in diameter. We reasoned that the extended PEG (DSPE-PEG) on the surface of the liposome would effectively protect the liposome from being attacked by blood components [33-36], and engulfed by phagocytes [37, 38] *in vivo*, thereby increasing the nonspecific tumor uptake. The transfection efficiency of SN in various human cancer cells was 2- to 5- fold higher than that of the most popular commercial transfection reagents, e.g. FuGene 6 (Roche, IN) and Lipofectamine (Invitrogen, MD). **We have also successfully demonstrated that the SN encapsulation of CMV-driven therapeutic genes can inhibit tumor growth *in vivo* through i.v. injection in both mammary and prostate tumor models** [10, 32, 39] (Appendix 1, 3, and 4).

1-4. BikDD is a potent therapeutic gene for gene therapy of prostate cancer.

1-4-1. Bik is a promising therapeutic gene. Bik (Bcl-2 interacting killer, also named as NBK, natural born killer), a pro-apoptotic gene in the Bcl-2 family that ultimately controls cell survival and death, efficiently kills various cancer cells through induction of apoptosis. Bik encodes an 18-kDa protein contains only the BH3 domain, which is essential for its pro-apoptotic activity [40-42]. Bik forms heterodimer with various anti-apoptotic proteins, e.g. Bcl-2 and Bcl-X_L [43-45]), inducing cytochrome c release without mitochondrial membrane potential loss [46, 47]]. Ectopic expression of Bik leads to decreased Ca^{+2} load [48]. It was recently shown that Bik might be induced by tumor hypoxia [49]. Loss of informative alleles on chromosome 22q where the Bik gene is located may be related to development of certain cancers, raising an interesting possibility that Bik may belong to the category of classical tumor suppressor gene [50] . In addition, Bik induces apoptosis through a p53-independent pathway [40]. Thus, Bik possesses many properties that allow for it to be an ideal therapeutic gene candidate for cancer treatment. **To support this notion, we have successfully demonstrated that the SN encapsulation of CMV-driven Bik, can inhibit tumor growth *in vivo* through i.v. injection in a mammary tumor model [32](Appendix 3)**

1-4-2. BikDD is more potent in antitumor activity than Bik. More recently, we further developed a Bik mutant (named BikDD), in which changes T33D and T35D were made to mimic the phosphorylation at these two

residues enhancing their binding affinity with the antiapoptotic proteins Bcl-X_L and Bcl-2 and were more potent than Bikwt and other Bcl-2 family pro-apoptotic genes, such as Bad, Bak and Bok, inducing apoptosis and inhibiting cell proliferation in various human cancer cells [39, 51]. BikDD-liposome complexes inhibited tumor growth and prolonged life span more effectively than the Bikwt-liposome complex in orthotopic animal models of breast cancer and s.c. prostate cancer xenografts (Appendix 4). **Thus, our results demonstrate that BikDD induces cell death (apoptosis) more potently than Bikwt, suggesting BikDD may be a very effective gene in a gene therapy setting for prostate cancer [39]** (Appendix 4).

1-4-3. BikDD may sensitize chemotherapy-induced apoptosis. Killing of tumor cells by diverse cytotoxic approaches such as anticancer drugs, γ-irradiation, suicide genes, or immunotherapy have been shown to involve induction of apoptosis in target cells [52-54]. A majority of human cancers including prostate cancer have been found to have overexpression of Bcl-2, Bcl-X_L, or both, suggesting that Bcl-2 and Bcl-X_L may play a critical role in cancer progression. Further, both clinical and laboratory studies have demonstrated that a high expression level of anti-apoptotic Bcl-2 proteins confers a wide spectrum of chemoresistant phenotypes on various cancer cells, including AML, ALL, CLL, breast carcinoma, prostate carcinoma, and malignant brain tumors [55-58]. Based on the Bcl-2 family's role in regulating mitochondrial/cytochrome c-mediated apoptosis and their frequently modified expression in human cancers, they are logical targets for the development of cancer chemotherapy by inducing apoptosis either by themselves or in association with other anticancer drugs. Consistent with this notion, Bik, which interacts with Bcl-2 or Bcl-X_L, has been shown not only to induce apoptosis of cancer cells but also sensitize chemoresistant cells to drug-induced apoptosis in multiple cancer types [59-61]. BikDD interacts with Bcl-2 and Bcl-X_L more strongerly and is more potent in killing cancer cells than Bikwt does [39] (Appendix 4). **It is reasonable to expect that BikDD will be more potent than Bikwt to sensitize chemotherapy-induced apoptosis. Thus, the potential sensitization effect by combining BikDD gene therapy with chemotherapy will be examined (Task 3).**

1-5. *In vivo* and real-time monitoring of tumor growth and metastasis of prostate cancer has been developed.

1-5-1. Bioluminescent imaging system is very useful for *in vivo* and real-time monitoring. Several small animal non-invasive, high-resolution imaging technologies are concurrently under development or being used that would be potentially useful for *in vivo* imaging [62]. The highest resolution approach, MRI, based on the differential behavior of electrons in various tissues, suffers from high cost, long sampling times and low sensitivity in detection of molecular probes [38]. Other relatively high resolution and penetrating techniques, such as PET and SPECT scans, are based on high-energy ionizing particles and are, thus, also expensive to carryout [63-65]. Some lower-cost techniques can provide very high sensitivity and higher throughput. A couple years ago, our lab established a bioluminescent imaging system, called the IVIS™ Xenogen system (Xenogen, Alameda, CA), based on charged-coupled device (CCD) camera, *in vivo* biophotonic imaging technology and computer science, which can effectively and safely measure light emitted from sources such as cells or small animals to track gene expression, the spread of a disease, or the effect of a new drug candidate *in vivo*. The co-PI, Dr. Xie, while working with Dr. David Spencer (Baylor College of Medicine), generated **an EZC**(easy see the using IVIS™ system)**-prostate transgenic mouse model in which a firefly luciferase gene was controlled by prostate-specific promoter hK2.E3/P [30]. The data demonstrate the real-time monitoring of the kinetics of prostate growth using this system** (Appendix 5). This animal model will be further explored in Task 2.

1-5-2. The IVIS™ Xenogen system has been used for *in vivo* and real-time monitoring of tumor growth and metastasis of prostate cancer in our lab. The bioluminescent imaging has been reported to detect lung and bone metastases of prostate tumors [66], to monitor growth of lymphoma tumors in abdomen in animal models [67], and detect *in vivo* cell proliferation and apoptosis [68, 69]. One advantage of this imaging technique is to detect metastatic tumors in a real-time manner without sacrificing the animals. Recently, we have established a glowing-prostate cancer orthotopic model in which the ventral part of the prostate was injected with the mouse-

derived RM-1-luc cell line that stably expresses luciferase under the control of the EF1a promoter. Using the IVIS™ Xenogen system, we can non-invasively and real-time monitor the orthotopic tumor growth and metastasis (Fig. 6). **In the current proposal, this non-invasive real-time imaging technique will be employed to enhance our ability to detect tumor sizes in response to gene therapy in animal studies.**

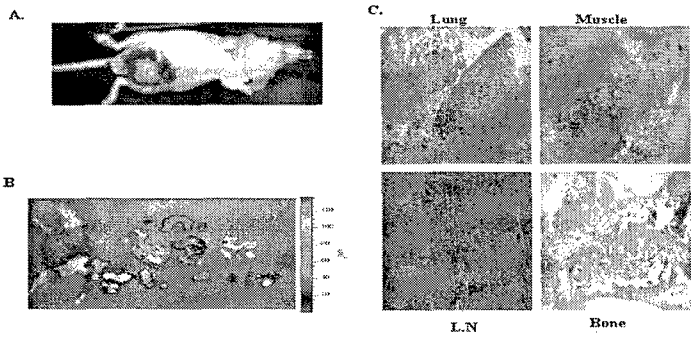

Fig. 4. EZC-prostate xenograft model. A. The in vivo imaging of the mice bearing orthtopic xenografts of RM-1-Luc cells. B. Six weeks later, the in vivo and ex vivo imaging shows tumor growth and metastasis. C. H.E. staining confirms the metastases in the lungs, muscle, lymph nodes (L.N) and bones.

2. HYPOTHESIS/PURPOSE

2-1. Hypothesis: As shown in the earlier section, the ATTP promoter highly demonstrates targeting transgene expression to ADPC and AIPC prostate cancer cells. BikDD possesses potent antitumor activity. And SN is a effectively systemic gene delivery system. The hypothesis is that ATTP-BikDD/SN is a powerful gene therapy agent for both ADPC and AIPC. This hypothesis will be rigorously tested in pre-clinical animal models in the current proposal. If successful, we will seek other funding for clinical trails in the future.

2-2. Rationale: ADPC is treated with ADT. However, this modality eventually fails because almost all of prostate cancer frequently develops to an androgen-independent state (AIPC). For these reasons, it is critical that a prostate cancer specific gene therapy functions not only in ADPC, but also in AIPC. ATTP is robust in prostate cancer cells and virtually silent in normal tissues, and its activity is comparable to the CMV promoter in AR^- prostate cancer, and 25-fold greater *in vitro* as well as 14.5-fold greater in LNCaP tumors *in vivo* than the CMV promoter (Fig. 2 and 3). Thus, ATTP-driven BikDD may effectively kill both ADPC and AIPC and should be minimum for potential side effect (compared with CMV-BikDD).

2-3: Purpose: To develop a novel therapeutic strategy for targeted gene therapy of ADPC and AIPC, not only in AR^+, but in AR^-, localized and metastasized prostate cancer. We will employ our recently developed non-viral delivery system (SN) to systemically deliver the ATTP-BikDD plasmid DNA, and real-time imaging system to monitor the kinetics of tumor growth and metastasis.

3. SPECIFIC AIMS

3-1. To test the targeted antitumor activity of ATTP-BikDD in ADPC and AIPC *in vitro* and in EZC-prostate cancer orthotopic models.

3.2. To test the targeted antitumor activity of ATTP-BikDD in ADPC and AIPC in an EZC-TRAMP model.

3.3. To evaluate the therapeutic efficiency of combined gene therapy with conventional chemotherapy.

4. RESEARCH DESIGN AND METHODS

4-1: Task 1: To test the targeted antitumor activity of ATTP-BikDD in ADPC and AIPC *in vitro* and in EZC- prostate cancer orthotopic models.

4-1-1: Generation and *in vitro* killing effect of ATTP-BikDD. We previously demonstrated that the expression of BikDD driven by the CMV promoter was more potent than Bikwt in inducing apoptosis and inhibiting cell proliferation in various human cancer cells including PC-3 and LNCaP cells, and suppressing tumorigenicity and tumor taking rate *ex vivo*. Further, we demonstrated that CMV-BikDD/SN complexes inhibited tumor growth and prolonged life span more effectively than CMV-Bikwt/SN complexes. In this proposal, using standard cloning methodology, we will generate a construct of ATTP-BikDD in the pUK21 context, which contains a kanamycin instead of ampicillin resistant gene, ultimately suitable for clinical trial use, . In addition, CMV-BikDD, ARR2-BikDD and ATTP alone in pUK21 will be used as controls. A panel of prostate cancer cell lines (LNCaP, PC-3, DU145, LAPC-4, TRAMP-C2 [TRAMP mouse-derived]), and normal primary human cells from prostate (PrEC, PrSC, PrSMC), and lung fibroblasts (WI-38), will be tested for apoptosis and cell proliferation inhibition mediated by these BikDD constructs. These primary cultured prostate cells were purchased (Combrex Bio Science, Inc., Baltimore). The cells will be cotransfected with BikDD plasmid, plus GL3.CMV-Luc. At 48 h posttransfection, the luciferase activity will be imaged for 2 min with the IVIS™ imaging system after 5 min incubation with 5 ng/ml of D-luciferin. The percentage of the relative signal in comparison to positive control (set at 100%) will be calculated. In addition, traditional methods (e.g. MTT, soft agar assays) will be used to assess the inhibition of proliferation. The TUNEL assay and Annex V staining and DNA fragmentation will also be explored to evaluate apoptosis. Western blotting will be used to examine the profiling of BikDD expression. Androgen analog R1881 will be used to see if it can stimulate or interfere with the activity.

Expected Results: ATTP will direct expression of BikDD to, and kill, all the prostate cancer cells tested. ATTP-BikDD will kill ADPC AR^+ (LNCaP) and AIPC AR^+ (LAPC-4) cells more efficiently than CMV-BikDD or ARR2PB-BikDD, and AIPC AR^- (PC-3 and DU145) cells at least comparable to CMV-BikDD, with minimal cytotoxicity to normal cells.

4-1-2: Establishment of EZC-prostate cancer orthotopic models. A more rigorous test for the effect of BikDD in a gene therapy setting is an orthotopic prostate tumor model through i.v. injection. In order to monitor in a real-time manner the growth and metastasis (if any) of prostate cancer, we first will establish prostate cancer cell lines that stably express firefly luciferase (LNCaP-Luc, PC-3-Luc and LAPC-4-Luc). This step is critical for real-time monitoring of the antitumor effect of ATTP-BikDD on prostate cancer in an orthotopic mouse models with the IVIS imaging system. The cell lines will be inoculated into the ventral part of the prostate of male nude mice as described previously [10, 30].

Expected Results: The tumor will be bioluminescent and easily seen (EZC) *in vivo* and real-time using our established imaging system (please see; Section 1-5-1 and 1-5-2) after an i.p. injection of D-luciferin. The mice will be monitored with the IVIS™ system to determine when the treatment begins.

4-1-3. Antitumor activity of ATTP-BikDD in EZC-prostate cancer orthotopic models. After the EZC-prostate cancer orthotopic models (LNCaP-Luc, PC-3-Luc, and LAPC-4-Luc) were established, the mice will be randomly divided into 4 groups, 10 mice per group. CMV-BikDD/SN, ATTP-BikDD/SN and ARR2PB-BikDD/SN, or ATTP/SN complexes will be systemically administrated 1-2 times per week for 5-10 weeks (a pre-test will be done). The potential therapeutic efficacy achieved from single injection and multiple injections (per week) will be compared. The kinetics of growth and metastasis (if any) of the tumor will be monitored by the real time imaging system. The tumor growth inhibition and the increased life span as well as systemic cytotoxicity will be evaluated. BikDD expression will be measured by *in situ* hybridization and immunohistochemical staining. The *in vivo* apoptosis index will be determined by TUNEL assay. Since the mice are expected to die within 5-8 weeks without treatment in this orthotopic model, the mice will be sacrificed when moribund and the survival time, prostate weight, and any paraaortic lymph node and distant metastases (also be ex vivo imagined to identify the metastasis) will be recorded. We will compare the therapeutic efficacy among the four groups.

Expected Results: The therapeutic efficacy will be observed in inhibition or eradication of xenograft tumor growth and metastasis of LNCaP-Luc, PC-3-Luc and LAPC-4-Luc, after treatment of CMV-BikDD/SN, ATTP-BikDD/SN. But there will be no therapeutic efficacy in PC-3-Luc tumors if treated with ARR2PB-BikDD/SN.

Furthermore, ATTP-BikDD/SN will be much more effective on LNCaP-Luc and LAPC-4-luc than ARR2PB-BikDD/SN and CMV-BikDD/SN. We expect that CMV-BikDD/SN through i.v. injection will produce BikDD expression, which will be widely distributed in various normal tissues such as heart, lung, liver, spleen and kidney. On the other hand, the expression of BikDD driven by ATTP will be predominately expressed in prostate tumors but not in normal tissues. In the case of ARR2PB-BikDD/SN, we expect to see a similar but much weaker expression pattern like ATTP-BikDD/SN, and with much less therapeutic efficacy than that of ATTP-BikDD/SN in LNCaP- and LAPC-4-induced tumors.

4-1-4. Antitumor activity of ATTP-BikDD in castrated mice. Androgen ablation by castration or biochemical antagonists represents the most frequently adopted or standard clinical procedures in the treatment of advanced prostate cancer patients. About 85 % of prostate (ADPC) will have an initial favorable response to this therapy [2]. However, over time molecular and cellular changes occur so that ADPC eventually progresses to AIPC. At this stage, the level of serum testosterone is often low. Therefore, it is critical to test whether ATTP-BikDD retains effective antitumor activity after androgen ablation. To accomplish this, the mice bearing AIPC tumors will be castrated following systemic administration of ATTP-BikDD. First, an orthotopic male nude mouse model will be established by inoculation of LAPC-4-Luc cells as described above. The tumor growth will be monitored with the IVIS™ system. Second, the mice will be castrated (this technique has mastered by our lab [10, 30]) when the tumor reaches an appropriate size. The tumor size will decrease due to decreased serum testosterone. Finally, when the tumor size starts increasing (often 3-4 weeks after castration) [70], the mice will be treated and data will be collected as described above.

Expected Results: After castration, the therapeutic efficacy of ATTP-BikDD/SN will be observed in inhibition or eradication of xenograft tumor growth including metastasis, of LAPC-4-Luc, comparable to or better than that of CMV-BikDD/SN based on results from Fig. 2B. This experiment has important clinical implication as many patients with AIPC have been castrated or androgen ablated.

4-2. Task 2: To test the targeted antitumor activity of ATTP-BikDD in ADPC and AIPC in an EZC-TRAMP model.

4-2-1. Establishment of an EZC-TRAMP model. Since spontaneous prostate cancer is primarily a disease of man, prostate cancer research initially relied on *in vitro* studies using human prostate cancer cell lines or *in vivo* studies using human xenografts in immunecompromised mice. However, these studies could not address the role of the immune system in limiting tumor growth and repeated plasmid administration. Additionally, cell lines may acquire mutations during *in vitro* culture that may decrease their relevance to human prostate cancer. Recently, the development of murine models for prostate cancer has facilitated the search for treatment options [7, 71]. Therefore, in this study, we will use the autochthonous TRAMP (transgenic adenocarcinoma of murine prostate) model [7], in which SV40 large T antigens are targeted to the mature epithelium of the dorsal, lateral and ventral acini, leading to progressive tumors in 100% of males between 18 and 24 weeks and metastasis within 28 weeks [7, 72]. Further, TRAMP-derived tumors are relatively slow growing, metastasize to draining lymph nodes, lungs and bone, and ultimately become increasingly androgen-insensitive, like the human disease [73, 74]. To develop a spontaneous prostate cancer model in which tumorigenicity and metastasis can be monitored with the IVIS™ system, we intent to create an EZC-TRAMP model by crossbreeding homozygous EZC-prostate mice with

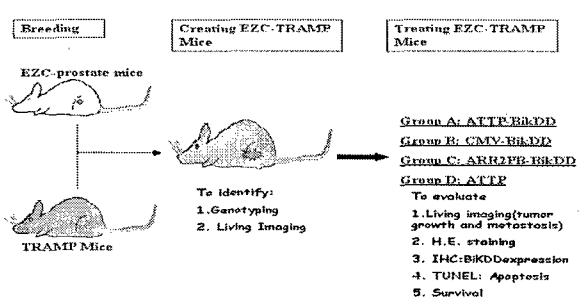

Fig. 5 Creation and treatment of EZC-TRAMP mice. EZC-prostate mice will be bred with TRAMP mice. The EZC-TRAMP mice will then be grouped and treated systemically and further evaluated for the antitumor effects among the groups with the IVIS system and traditional methods.

homozygous TRAMP mice. The bigenic (i.e. carrying both luciferase and SV40 large T antigens) mice, EZC-TRAMP (Fig. 5) will be genotyped by polymerase chain reaction and examined for bioluminescence with the IVIS™ system. If this animal model is obtained, it will be very useful to tempo-spatially monitor tumor incidence and follow-up therapeutic efficacy by in vivo imaging.

Expected Results: The growth kinetics and metastases of the tumors in the EZC-TRAMP mice can be monitored with the IVIS system. If the EZC-TRAMP prostate cancer animal model is established, it will be very helpful in identifying mice bearing spontaneous tumor for treatment.

4-2-2. Antitumor activity of ATTP-BikDD/SN in the EZC-TRAMP model. Most TRAMP mice start hyperplasia with nuclear atypia at 10 weeks of age, and display invasive adenocarcinoma at 16 weeks of age. Mature 10-and 16-week-old EZC-TRAMP mice (n=5 per group) will be i.v. injected with CMV-BikDD/SN, ATTP-BikDD/SN, ARR2PB-BikDD/SN, or ATTP/SN complexes 1-2 times per week for up to 8-10 weeks. The kinetics of growth and metastasis (if any) of the tumor will be monitored by the real time imaging system, and BikDD expression, the *in vivo* apoptosis index, and morphologic changes will be determined described above in the midst of treatment. AR expression will also be examined. The mice will be sacrificed when moribund and the survival time, prostate weight, and any paraaortic lymph node metastasis will be recorded as described.

Expected Results: The therapeutic efficacy will lead to inhibition or eradication of tumor growth and metastasis after treatment of CMV-BikDD/SN, ATTP-BikDD/SN and ARR2PB-BikDD/SN. ATTP-BikDD/SN will be much more effective than CMV-BikDD/SN and ARR2PB-BikDD/SN. Similar to the orthotopic models, CMV-BikDD/SN, through i.v. injection, will produce BikDD expression which will be widely distributed in various normal tissues such as heart, lung, liver, spleen and kidney. However, the expression of BikDD driven by the ATTP will be predominately expressed in tumors but not in normal tissues. ARR2PB will direct BikDD expression to normal prostate tissue and prostate cancer tissue. Since both EZC-prostate mice and TRAMP mice are available, it is feasible to generate EZC-TRAMP mice for the above experiment. However, in case the tumor in the EZC-TRAMP mice is not bioluminescent as expected, we can still use TRAMP mice to perform these experiments.

4-3. Task 3. To evaluate the therapeutic efficiency of combined gene therapy with conventional chemotherapy.

4-3-1. Therapeutic efficiency of combined gene therapy with conventional chemotherapy in vitro. We will examine the potential synergistic/additive effect on the killing of prostate cancer cells in cell culture of BikDD overexpression in combination with chemotherapy treatment. Among chemo-agents, we will be particularly interested in those agents such as docetaxel, paclitaxel, doxorubicin, and cisplatin [43, 75], which are commonly administered to patients with advanced prostate cancer [75]. Prostate cancer cell lines will be transfected with ATTP-BikDD, then treated with different dosages of chemo-agents. AR^+ (LNCaP, LAPC-4) and AR^- (PC-3 and DU145) prostate cancer lines in our own laboratory will be investigated. Specifically, we will focus on the LAPC-4 cells due to its AR^+ and AIPC characterization. To ensure that we have a large window to measure chemotherapy-induced apoptosis, we will first determine the dosage of ATTP-BikDD/SN that can induce at least 5-10% killing of the transfected prostate cancer cells in the absence of chemo-agents. Once the dosage of ATTP-BikDD is determined for each prostate cancer cell line, it will be combined with a wide range of chemo-agents to obtain the accurate IC_{50}. The IC_{50} of each chemo-agent will be compared in the presence/absence of ATTP-BikDD transfection. The relative cell survival will be measured by cell number counting and MTT assays which will be used to calculate the IC_{50}; and Annexin V, TUNEL and FACS analyses in several selected doses will confirm the apoptosis.

Expected Results: Since Bik has been shown to sensitize chemo-induced cell killing in other cancer cells, we expect that BikDD, which interacts with Bcl2 and Bcl-X_L more stongly than Bikwt, is likely to sensitize cells to chemotherapy in culture. Therefore, the dosage of IC_{50} for each chemo-agent in the presence of BikDD is expected to be much lower than those in the absence of BikDD. The chemo-agents that show a significantly lower IC_{50} in the presence of BikDD, will be selected for future tests in the combined therapy *in vivo*.

4-3-2. Therapeutic efficiency of combined gene therapy with conventional chemotherapy in vivo. In this setting, chemotherapy can be administered by i.p or i.v. injection and gene therapy (ATTP-BikDD) will be administered by i.v. injection in the models described in Task 1 and Task 2. We are familiar with the combination of chemotherapy and gene therapy as described in our previous publication [52]. Both tumor growth and survival rate will be measured as described as above. To ensure that a maximum sensitization effect will be found, the dosage of ATTP-BikDD/SN and chemo-agents will be optimized.

Expected Results: Some chemo-agents will show synergistic or additive therapeutic effect of ATTP-BikDD/SN on prostate cancer *in vivo*. If successful, it will provide substantial evidence for further clinical trails.

5. Discussion, Alternative Approaches and Possible Pitfalls.

The proposal is based on the encouraging preliminary results showing that 1) BikDD possesses anti-tumor activity in multiple cancer cell types, including prostate cancer cells *in vitro* and *in vivo*, 2) ATTP strongly targets the transgene expression to the ADPC and AIPC, AR^+ and AR^- prostate cancer cells, 3) SN is an effective systemic delivery system for gene therapy, and 4) *In vivo* and real-time monitoring of tumor growth and metastasis of prostate cancer has been developed in our lab. Almost all the proposed experiments such as transfection, growth assay, systemic delivery, TUNEL assay, *in vivo* and ex vivo imaging, orthotopic model, transgenic mouse model, castration operation, preclinical gene therapy experiments, *in vivo* imaging, DNA/SN complex, etc. are well-established techniques in the PI's laboratory as shown in the preliminary results or PI and co-PI's publications (please see attached appendix). Thus, the techniques in this proposal should not be a problem. We expect that within the three-year funding period, we should be able to develop a potential novel therapeutic strategy using ATTP-BikDD gene therapy in combination with some chemotherapies in the preclinical setting which, if successful, should enable us to translate it into clinical trials. We realize the fact that both viral and non-viral gene delivery systems have their own strengths and weaknesses. We have successfully used the non-viral delivery system in gene therapy starting from preclinical settings to clinical trials[76]. We feel that it would be more productive for us to use what we are good at, i.e., BikDD/liposome. Therefore, non-viral delivery gene therapy will be focused on in this proposal. However, if other gene delivery system is available and shown to be a better approach, we will also explore the possibility of using it in this project.

Another concern is that the ATTP promoter that contains the TSTA system, leads to expression of a non-human protein derived from Gal4VP2, which is a fusion protein linking a Gal4 binding domain to a transcriptional activator domain of VP16. This non-human protein, if expressed in normal cells, might be immunogenic and induce unforeseen potential adversary effects. However, since it is driven by the prostate cancer-specific promoter (ATTP), Gal4VP2 will be predominately expressed in prostate cancer cells. Thus, the potential adversary effects should be minimal (Task 2). As a matter of fact, if it is immunogenic and expressed in the prostate cancer cells, it could be an advantage from a therapeutic point of view as it may facilitate a host immune response to kill the prostate cancer cells expressing this protein. All these safety issues should be experimentally tested in the standard toxicity study in the future before the initiation of a clinical trial.

Abbreviations

ADIC androgen-independent prostate cancer
ADPC androgen-dependent prostate cancer
ADPC androgen-dependent prostate cancer
ADT androgen deprivation therapy
AIPC androgen-independent prostate cancer
AR androgen receptor
ARR2 androgen receptor responsive element 2
ARR2PB a modified PB promoter that directs a high prostate cancer-specific gene
ATT a promoter composite comtaining ARR2, hTERTp and TSTA
ATTP a promoter composite comtaining ARR2, hTERTp, TSTA and WPRE
Bik or Bikwt Bcl-2 interacting killer, also named as NBK, natural born killer
BikDD a Bik mutant (T33D, S35D)
CMV cytomegalovirus
D-luciferin a substrate for Luc luciferase
EZC easy see (using the IVIS™ system)
FACS fluorescence-activated cell sorter
G418 neomycin (or Genecin)
hK2 human kallikrein 2
hK2-E3/P hK2 promoter containg 3 enhancers.
HTERT human telomerase reverse transcriptase
hTERTp human telomerase reverse transcriptase promoter
HUVEC human umbilical vein endothelial cells
i.p. intraperitoneal(ly)r
i.v. intravenous(ly)
IVIS™ an trademark of Xenogen, Inc., meaning *In Vivo* Imaging System.
LAPC-4 human AR^+ prostate cancer cell line
LNCaP human AR^+ prostate cancer cell line
MRI magnetic resonance imaging
MTT 3, (4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide
PB probasin
PC3, DU145 human AR^- prostate cancer cell line
PET positron emission tomography
PrEC prostate epithelial cell
PrSC prostate stromal cells
PrSMC prostate smooth muscle cells
PSMA prostate-specific membrane antigen
s.c. subcutaneous(ly)
SN stabilized non-viral liposme-based delivery system.
SPECT single photon emission computed tomography
TRAMP transgenic adenocarcinoma of murine prostate
TSTA two-steps transcriptional amplification
TUNEL terminal deoxynucleotidyl transferase dUTP nick end labeling
WPRE woodchuck hepatitis virus response enhances

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